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(71) Applicant: **METHYLGENE, INC.** [CA/CA]; 7220 Frederick-Banting, St. Laurent, Quebec H4S 2A1 (CA).

(72) Inventors: **LI, Zuomei**; 22 Oriole Street, Kirkland, H9H 3x3 (CA). **BONFILS, Claire**; 10629 Rue St. Hubert, Montreal, Quebec H9X 3V3 (CA). **BESTERMAN, Jeffrey**; 51 Gray Crescent, Baie d'Urfe, H9X 3V3 (CA).

(74) Agents: **COTE, France** et al.; Swabey Ogilvy Renault, 1981 McGill College Ave. - Suite 1600, Montréal, Québec H3A 2Y3 (CA).

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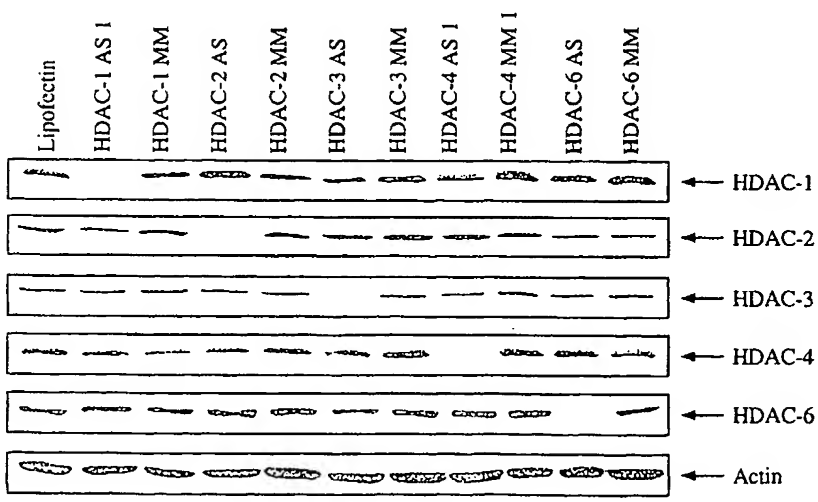
(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS



AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc – 50nM

(57) **Abstract:** This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

WO 03/006652 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the fields of inhibition of histone deacetylase expression and enzymatic activity.

Summary of the Related Art

In eukaryotic cells, nuclear DNA associates with histones to form a compact complex called chromatin. The histones constitute a family of basic proteins which are generally highly conserved across eukaryotic species. The core histones, termed H2A, H2B, H3, and H4, associate to form a protein core. DNA winds around this protein core, with the basic amino acids of the histones interacting with the negatively charged phosphate groups of the DNA. Approximately 146 base pairs of DNA wrap around a histone core to make up a nucleosome particle, the repeating structural motif of chromatin.

Csordas, *Biochem. J.*, 286: 23-38 (1990) teaches that histones are subject to posttranslational acetylation of the epsilon-amino groups of *N*-terminal lysine residues, a reaction that is catalyzed by histone acetyl transferase (HAT1). Acetylation neutralizes the positive charge of the lysine side chain, and is thought to impact chromatin structure. Indeed, Taunton *et al.*, *Science*, 272: 408-411 (1996), teaches that access of transcription factors to chromatin templates is enhanced by histone hyperacetylation. Taunton *et al.* further teaches that an enrichment in underacetylated histone H4 has been found in transcriptionally silent regions of the genome.

Recently, there has been interest in the role of histone deacetylase (HDAC) in gene expression. Sanches Del Pino *et al., Biochem. J.* 303: 723-729 (1994) discloses a partially purified yeast HDAC activity. Taunton *et al. (supra)* discloses a human HDAC that is related to a yeast transcriptional regulator and suggests that this protein may be a key regulator of eukaryotic transcription.

Known inhibitors of mammalian HDAC have been used to probe the role of HDAC in gene regulation. Yoshida *et al., J. Biol. Chem* 265: 17174-17179 (1990) discloses that (R)-Trichostatin A (TSA) is a potent inhibitor of mammalian HDAC. Yoshida *et al, Cancer Research* 47: 3688-3691 (1987) discloses that TSA is a potent inducer of differentiation in murine erythroleukemia cells.

More recently, it has been discovered that the HDAC activity is actually provided by a set of discrete HDAC enzyme isoforms. Grozinger *et al., Proc. Natl. Acad. Sci. (USA),* 96: 4868-4873 (1999), teaches that HDACs may be divided into two classes, the first represented by yeast Rpd3-like proteins, and the second represented by yeast Hda1-like proteins. Grozinger *et al.* also teaches that the human HDAC1, HDAC2, and HDAC3 proteins are members of the first class of HDACs, and discloses new proteins, named HDAC4, HDAC5, and HDAC6, which are members of the second class of HDACs. Kao *et al., Gene & Development* 14: 55-66 (2000), discloses an additional member of this second class, called HDAC-7. More recently, Hu, E. *et al. J. Bio. Chem.* 275:15254-13264 (2000) disclosed the newest member of the first class of histone deacetylases, HDAC-8. It has been unclear what roles these individual HDAC enzymes play.

The known inhibitors of histone deacetylase are all small molecules that inhibit histone deacetylase activity at the protein level. Moreover, all of the known histone deacetylase inhibitors are non-specific for a particular histone deacetylase isoform, and more or less inhibit all members of both the histone deacetylase families equally.

Therefore, there remains a need to develop reagents for inhibiting specific histone deacetylase isoforms. There is also a need for the development of methods for using these reagents to identify and inhibit specific histone deacetylase isoforms involved in tumorigenesis.

BRIEF SUMMARY OF THE INVENTION

The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level. The invention allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative and/or differentiation disorders.

The inventors have discovered new agents that inhibit specific HDAC isoforms. Accordingly, in a first aspect, the invention provides agents that inhibit one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms. Such specific HDAC isoforms include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. Non-limiting examples of the new agents include antisense oligonucleotides (oligos) and small molecule inhibitors specific for one or more HDAC isoforms but less than all HDAC isoforms.

The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding that histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA. In some embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC isoform. In other embodiments, the oligonucleotide inhibits translation of the histone deacetylase isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Particularly preferred embodiments include antisense oligonucleotides directed to HDAC-1 and/or HDAC-4.

In yet other embodiments of the first aspect, the agent that inhibits a specific HDAC isoform is a small molecule inhibitor that inhibits the activity of one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms.

In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell, comprising contacting the cell with an agent of the first aspect of the invention. In other preferred embodiments, the agent is an antisense oligonucleotide. In certain preferred embodiments, the agent is a small molecule inhibitor. In other certain preferred embodiments of the second aspect of the invention, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method of the second aspect of the invention further comprises contacting the cell with a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of one or more specific histone deacetylase isoforms. In still yet other preferred embodiments of the second aspect of the invention, the method comprises an agent of the first aspect of the invention which is a

combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4. In some embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain preferred embodiments, the agent is a small molecule inhibitor which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain preferred embodiments of the this aspect of the invention, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In other certain embodiments, the agent is a small molecule inhibitor of the first aspect of the invention which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In still yet other preferred embodiments of the third aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred

embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is an small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time.

In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide from the first aspect of the invention that inhibits expression of a specific histone deacetylase isoform, a small molecule inhibitor from the first aspect of the invention that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a small molecule that inhibits a DNA methyltransferase. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation, comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In certain embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In preferred embodiments, the cell proliferation is neoplasia. In still yet other preferred embodiments of the this aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic diagram providing the amino acid sequence of HDAC-1, as provided in GenBank Accession No. AAC50475 (SEQ ID NO:1).

Figure 1B is a schematic diagram providing the nucleic acid sequence of HDAC-1, as provided in GenBank Accession No. U50079 (SEQ ID NO:2).

Figure 2A is a schematic diagram providing the amino acid sequence of HDAC-2, as provided in GenBank Accession No. AAC50814 (SEQ ID NO:3).

Figure 2B is a schematic diagram providing the nucleic acid sequence of HDAC-2, as provided in GenBank Accession No. U31814 (SEQ ID NO:4).

Figure 3A is a schematic diagram providing the amino acid sequence of HDAC-3, as provided in GenBank Accession No. AAB88241 (SEQ ID NO:5).

Figure 3B is a schematic diagram providing the nucleic acid sequence of HDAC-3, as provided in GenBank Accession No. U75697 (SEQ ID NO:6).

Figure 4A is a schematic diagram providing the amino acid sequence of HDAC-4, as provided in GenBank Accession No. BAA22957 (SEQ ID NO:7).

Figure 4B is a schematic diagram providing the nucleic acid sequence of HDAC-4, as provided in GenBank Accession No. AB006626 (SEQ ID NO:8).

Figure 5A is a schematic diagram providing the amino acid sequence of HDAC-5, as provided in GenBank Accession No. BAA25526 (SEQ ID NO:9).

Figure 5B is a schematic diagram providing the nucleic acid sequence of HDAC-5 as provided in GenBank Accession No. AB011172 (SEQ ID NO:10).

Figure 6A is a schematic diagram providing the amino acid sequence of human HDAC-6, as provided in GenBank Accession No. AAD29048 (SEQ ID NO:11).

Figure 6B is a schematic diagram providing the nucleic acid sequence of human HDAC-6, as provided in GenBank Accession No. AJ011972 (SEQ ID NO:12).

Figure 7A is a schematic diagram providing the amino acid sequence of human HDAC-7, as provided in GenBank Accession No. AAF63491.1 (SEQ ID NO:13).

Figure 7B is a schematic diagram providing the nucleic acid sequence of human HDAC-7, as provided in GenBank Accession No. AF239243 (SEQ ID NO:14).

Figure 8A is a schematic diagram providing the amino acid sequence of human HDAC-8, as provided in GenBank Accession No. AAF73076.1 (SEQ ID NO:15).

Figure 8B is a schematic diagram providing the nucleic acid sequence of human HDAC-8, as provided in GenBank Accession No. AF230097 (SEQ ID NO:16).

Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-1 AS1 antisense oligonucleotide on HDAC-1 mRNA expression in human A549 cells.

Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-2 AS antisense oligonucleotide on HDAC-2 mRNA expression in human A549 cells.

Figure 9C is a representation of a Northern blot demonstrating the effect of HDAC-6 AS antisense oligonucleotide on HDAC-6 mRNA expression in human A549 cells.

Figure 9D is a representation of a Northern blot demonstrating the effect of HDAC-3 AS antisense oligonucleotide on HDAC-3 mRNA expression in human A549 cells.

Figure 9E is a representation of a Northern blot demonstrating the effect of an HDAC-4 antisense oligonucleotide (AS1) on HDAC-4 mRNA expression in human A549 cells.

Figure 9F is a representation of a Northern blot demonstrating the dose-dependent effect of an HDAC-4 antisense oligonucleotide (AS2) on HDAC-4 mRNA expression in human A549 cells.

Figure 9G is a representation of a Northern blot demonstrating the effect of an HDAC-5 antisense oligonucleotide (AS) on HDAC-5 mRNA expression in human A549 cells.

Figure 9H is a representation of a Northern blot demonstrating the effect of an HDAC-7 antisense oligonucleotide (AS) on HDAC-7 mRNA expression in human A549 cells.

Figure 9I is a representation of a Northern blot demonstrating the dose-dependent effect of HDAC-8 antisense oligonucleotides (AS1 and AS2) on HDAC-8 mRNA expression in human A549 cells.

Figure 10A is a representation of a Western blot demonstrating the effect of HDAC isotype-specific antisense oligos on HDAC isotype protein expression in human A549 cells.

Figure 10B is a representation of a Western blot demonstrating the dose-dependent effect of the HDAC-1 isotype-specific antisense oligo (AS1 and AS2) on HDAC isotype protein expression in human A549 cells.

Figure 10C is a representation of a Western blot demonstrating the effect of HDAC-4 isotype-specific antisense oligonucleotide (AS2) on HDAC isotype protein expression in human A549 cells.

Figure 11A is a graphic representation demonstrating the apoptotic effect of HDAC isotype-specific antisense oligos on human A549 cancer cells.

Figure 12A is a graphic representation demonstrating the effect of HDAC-1 AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

Figure 12B is a graphic representation demonstrating the effect of HDAC-8 specific AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

Figure 13 is a a graphic representation demonstrating the cell cycle blocking effect of HDAC specific antisense oligonucleotides on human A549 cancer cells.

Figure 14 is a representation of an RNAse protection assay demonstrating the effect of HDAC isotype-specific antisense oligonucleotides on HDAC isotype mRNA expression in human A549 cells.

Figure 15 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-4 AS1 antisense oligonucleotide induces the expression of the p21 protein.

Figure 16 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-1 antisense oligonucleotides (AS1 and AS2) represses the expression of the cyclin B1 and cyclin A genes.

Figure 17 shows plating data demonstrating the ability of antisense oligonucleotides complementary to HDAC-1 to inhibit growth in soft agar of A549 cells far more than can antisense oligonucleotides complementary to HDAC-2, HDAC-6 or mismatched controls.

Figure 18 is a representation of a Western blot demonstrating that treatment of human A549 cells with the small molecule inhibitor Compound 3 (Table 2) induces the expression of the p21 protein and represses the expression of the cyclin B1 and cyclin A genes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides methods and reagents for inhibiting specific histone deacetylase isoforms (HDAC) by inhibiting expression at the nucleic acid level or protein activity at the enzymatic level. The invention allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative and/or differentiation disorders.

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

In a first aspect, the invention provides agents that inhibit one or more histone deacetylase isoform, but less than all specific histone deacetylase isoforms. As used herein interchangeably, the terms "histone deacetylase", "HDAC", "histone deacetylase isoform", "HDAC isoform" and similar terms are intended to refer to any one of a family of enzymes that remove acetyl groups from the epsilon-amino groups of lysine residues at the *N*-terminus of a histone. Unless otherwise indicated by context, the term "histone" is meant to refer to any histone protein, including H1, H2A, H2B, H3, and H4, from any species. Preferred histone deacetylase isoforms include class I and class II enzymes. Specific HDACs include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. By way of non-limiting example, useful agents that

inhibit one or more histone deacetylase isoforms, but less than all specific histone deacetylase isoforms, include antisense oligonucleotides and small molecule inhibitors.

The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

Preferred agents that inhibit HDAC-1 and/or HDAC-4 dramatically inhibit growth of human cancer cells, independent of p53 status. These agents significantly induce apoptosis in the cancer cells and cause dramatic growth arrest. They also can induce transcription of tumor suppressor genes, such as $p21^{WAF1}$, $p57^{KIP2}$, GADD153 and GADD45. Finally, they exhibit both *in vitro* and *in vivo* anti-tumor activity. Inhibitory agents that achieve one or more of these results are considered within the scope of this aspect of the invention. By way of non-limiting example, antisense oligonucleotides and/or small molecule inhibitors of HDAC-1 and/or HDAC-4 are useful for the invention.

In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding a specific histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA. In other embodiments, the oligonucleotide ultimately inhibits translation of the histone deacetylase. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Preferred antisense

oligonucleotides have potent and specific antisense activity at nanomolar concentrations.

The antisense oligonucleotides according to the invention are complementary to a region of RNA or double-stranded DNA that encodes a portion of one or more histone deacetylase isoform (taking into account that homology between different isoforms may allow a single antisense oligonucleotide to be complementary to a portion of more than one isoform).

For purposes of the invention, the term "complementary" means having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base stacking can lead to hybridization. As a practical matter, such hybridization can be inferred from the observation of specific gene expression inhibition, which may be at the level of transcription or translation (or both).

For purposes of the invention, the term "oligonucleotide" includes polymers of two or more deoxyribonucleosides, ribonucleosides, or 2'-O-substituted ribonucleoside residues, or any combination thereof. Preferably, such oligonucleotides have from about 8 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The nucleoside residues may be coupled to each other by any of the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate,

carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleotide linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines, and adamantane. The term oligonucleotide also encompasses such polymers as PNA and LNA. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with an -O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl, or allyl group may be unsubstituted or may be substituted, *e.g.*, with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group.

Particularly preferred antisense oligonucleotides utilized in this aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or phosphorodithioate region, preferably comprising from about 2 to about 12 nucleotides, and an alkylphosphonate or alkylphosphonothioate region (see *e.g.*, Pederson *et al.* U.S. Patent Nos. 5,635,377 and 5,366,878). Preferably, such chimeric oligonucleotides contain at least three consecutive internucleoside linkages selected from phosphodiester and phosphorothioate linkages, or combinations thereof.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred embodiment of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-O-substituted ribonucleotide region, preferably comprising from about 2 to about 12 2'-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide will contain at least three consecutive deoxyribonucleosides and will also contain ribonucleosides, 2'-O-substituted ribonucleosides, or combinations thereof (see *e.g.*, Metelev and Agrawal, U.S. Patents Nos. 5,652,355 and 5,652,356).

The exact nucleotide sequence and chemical structure of an antisense oligonucleotide utilized in the invention can be varied, so long as the oligonucleotide retains its ability to inhibit expression of a specific histone deacetylase isoform or inhibit one or more histone deacetylase isoforms, but less than all specific histone deacetylase isoforms. This is readily determined by testing whether the particular antisense oligonucleotide is active by quantitating the amount of mRNA encoding a specific histone deacetylase isoform, quantitating the amount of histone

deacetylase isoform protein, quantitating the histone deacetylase isoform enzymatic activity, or quantitating the ability of the histone deacetylase isoform to inhibit cell growth in a an *in vitro* or *in vivo* cell growth assay, all of which are described in detail in this specification. The term "inhibit expression" and similar terms used herein are intended to encompass any one or more of these parameters.

Antisense oligonucleotides utilized in the invention may conveniently be synthesized on a suitable solid support using well-known chemical approaches, including H-phosphonate chemistry, phosphoramidite chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase oligonucleotide synthesis, such as controlled-pore glass (CPG) (see, *e.g.*, Pon, R. T., Methods in Molec. Biol. 20: 465-496, 1993).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of specific histone deacetylase isoforms by being used to inhibit the activity of specific histone deacetylase isoforms in an experimental cell culture or animal system and to evaluate the effect of inhibiting such specific histone deacetylase isoform activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide that inhibits one or more histone deacetylase isoform expression according to the invention and observing any phenotypic effects. In this use, the antisense oligonucleotides according to the invention is preferable to traditional "gene knockout" approaches because it is easier to use, and can be used to inhibit specific histone deacetylase isoform activity at selected stages of development or differentiation.

Preferred antisense oligonucleotides of the invention inhibit either the transcription of a nucleic acid molecule encoding the histone deacetylase isoform, and/or the translation of a nucleic acid molecule encoding the histone deacetylase isoform, and/or lead to the degradation of such nucleic acid. Histone deacetylase-encoding nucleic acids may be RNA or double stranded DNA regions and include, without limitation, intronic sequences, untranslated 5′ and 3′ regions, intron-exon boundaries as well as coding sequences from a histone deacetylase family member gene. For human sequences, see *e.g.*, Yang et al., *Proc. Natl. Acad. Sci. (USA)* 93(23): 12845-12850, 1996; Furukawa et al., *Cytogenet. Cell Genet.* 73(1-2): 130-133, 1996; Yang et al., *J. Biol. Chem.* 272(44): 28001-28007, 1997; Betz et al., *Genomics* 52(2): 245-246, 1998; Taunton et al., *Science* 272(5260): 408-411, 1996; and Dangond et al., *Biochem. Biophys. Res. Commun.* 242(3): 648-652, 1998).

Particularly preferred non-limiting examples of antisense oligonucleotides of the invention are complementary to regions of RNA or double-stranded DNA encoding a histone deacetylase isoform (*e.g.*, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8). (see *e.g.*, GenBank Accession No. U50079 for human HDAC-1 (Fig. 1B); GenBank Accession No. U31814 for human HDAC-2; (Fig. 2B) GenBank Accession No. U75697 for human HDAC-3 (Fig. 3B; GenBank Accession No. AB006626 for human HDAC-4 (Fig. 4B); GenBank Accession No. AB011172 for human HDAC-5 (Fig. 5B); GenBank Accession No. AJ011972 for human HDAC-6 (Fig. 6B); GenBank Accession No. AF239243 for human HDAC-7 (Fig. 7B); and GenBank Accession No. AF230097 for human HDAC-8 (Fig. 8B)).

The sequences encoding histone deacetylases from many non-human animal species are also known (see, for example, GenBank Accession Numbers X98207 (murine HDAC-1); NM_008229 (murine HDAC-2); NM_010411 (murine HDAC-3); NM_006037 (murine HDAC-4); NM_010412 (murine HDAC-5); NM_010413 (murine HDAC-6); and AF207749 (murine HDAC-7)). Accordingly, the antisense oligonucleotides of the invention may also be complementary to regions of RNA or double-stranded DNA that encode histone deacetylases from non-human animals. Antisense oligonucleotides according to these embodiments are useful as tools in animal models for studying the role of specific histone deacetylase isoforms.

Particularly, preferred oligonucleotides have nucleotide sequences of from about 13 to about 35 nucleotides which include the nucleotide sequences shown in Table I. Yet additional particularly preferred oligonucleotides have nucleotide sequences of from about 15 to about 26 nucleotides of the nucleotide sequences shown below. Most preferably, the oligonucleotides shown below have phosphorothioate backbones, are 20-26 nucleotides in length, and are modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2' -O- methyl groups attached to their sugar residues.

Antisense oligonucleotides used in the present study are shown in Table I.

Table 1

Sequences of Human Isotype-Specific Antisense (AS) Oligonucleotides and Their Mismatch (MM) Oligonucleotides

Oligo	Target	Accession Number	Nucleotide Position	Sequence	Gene Position
HDAC1 AS1	Human HDAC1	U50079	1585-1604	5'-GAAACGTGAGGGACTCAGCA-3' (SEQ ID NO:17)	3'-UTR
HDAC1 AS2	Human HDAC1	U50079	1565-1584	5'-GGAAGCCAGAGCTGGAGAGG-3' (SEQ ID NO:18)	3'-UTR
HDAC1 MM	Human HDAC1	U50079	1585-1604	5'-GTTAGGTGAGGCACTGAGGA-3' (SEQ ID NO:19)	3'-UTR
HDAC2 AS	Human HDAC2	U31814	1643-1622	5'-GCTGAGCTGTTCTGATTTGG-3' (SEQ ID NO:20)	3'-UTR
HDAC2 MM	Human HDAC2	U31814	1643-1622	5'-CGTGAGCACTTCTCATTTCC-3' (SEQ ID NO:21)	3'-UTR
HDAC3 AS	Human HDAC3	AF039703	1276-1295	5'-CGCTTTCCTTGTCATTGACA-3' (SEQ ID NO:22)	3'-UTR
HDAC3 MM	Human HDAC3	AF039703	1276-1295	5'-GCCTTTCCTACTCATTGTGT-3' (SEQ ID NO:23)	3'-UTR
HDAC4 AS1	Human HDAC4	AB006626	514-33	5-GCTGCCTGCCGTGCCCACCC-3' (SEQ ID NO:24)	5'-UTR
HDAC4 MM1	Human HDAC4	AB006626	514-33	5'-CGTGCCTGCGCTGCCCACGG-3' (SEQ ID NO:25)	5'-UTR
HDAC4 AS2	Human HDAC4	AB006626	7710-29	5'-TACAGTCCATGCAACCTCCA-3' (SEQ ID NO:26)	3'-UTR
HDAC4 MM4	Human HDAC4	AB006626	7710-29	5'-ATCAGTCCAACCAACCTCGT-3' (SEQ ID NO:27)	3'-UTR
HDAC5 AS	Human HDAC5	AF039691	2663-2682	5'-CTTCGGTCTCACCTGCTTGG-3' (SEQ ID NO:28)	3'-UTR
HDAC6 AS	Human HDAC6	AJ011972	3791-3810	5'-CAGGCTGGAATGAGCTACAG-3' (SEQ ID NO:29)	3'-UTR
HDAC6 MM	Human HDAC6	AJ011972	3791-3810	5'-GACGCTGCAATCAGGTAGAC-3' (SEQ ID NO:30)	3'-UTR
HDAC7 AS	Human HDAC7	AF239243	2896-2915	5'-CTTCAGCCAGGATGCCCACA-3' (SEQ ID NO:31)	3'-UTR
HDAC8 AS1	Human HDAC8	AF230097	51-70	5'-CTCCGGCTCCTCCATCTTCC-3' (SEQ ID NO:32)	5'-UTR
HDAC8 AS2	Human HDAC8	AF230097	1328-1347	5'-AGCCAGCTGCCACTTGATGC-3' (SEQ ID NO:33)	3'-UTR

The antisense oligonucleotides according to the invention may optionally be formulated with any of the well known pharmaceutically acceptable carriers or diluents (see preparation of pharmaceutically acceptable formulations in, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990), with the proviso that such carriers or diluents not affect their ability to modulate HDAC activity.

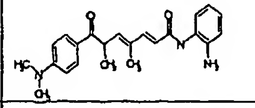
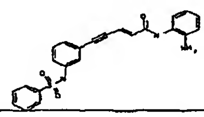
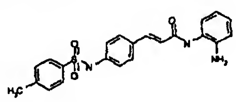
By way of non-limiting example, the agent of the first aspect of the invention may also be a small molecule inhibitor. The term "small molecule" as used in reference to the inhibition of histone deacetylase is used to identify a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably less than 600 Da, which is capable of interacting with a histone deacetylase and inhibiting the expression of a nucleic acid molecule encoding an HDAC isoform or activity of an HDAC protein. Inhibiting histone deacetylase enzymatic activity means reducing the ability of a histone deacetylase to remove an acetyl group from a histone. In some preferred embodiments, such reduction of histone deacetylase activity is at least about 50%, more preferably at least about 75%, and still more preferably at least about 90%. In other preferred embodiments, histone deacetylase activity is reduced by at least 95% and more preferably by at least 99%. In one certain embodiment, the small molecule inhibitor is an inhibitor of one or more but less than all HDAC isoforms. By "all HDAC isoforms" is meant all proteins that specifically remove an epsilon acetyl group from an N-terminal lysine of a histone, and includes, without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8, all of which are considered "related proteins," as used herein.

Most preferably, a histone deacetylase small molecule inhibitor interacts with and reduces the activity of one or more histone deacetylase isoforms (*e.g.*, HDAC-1 and/or HDAC-4), but does not interact with or reduce the activities of all of the other histone deacetylase isoforms (*e.g.*, HDAC-2 and HDAC-6). As discussed below, a preferred histone deacetylase small molecule inhibitor is one that interacts with and reduces the enzymatic activity of a histone deacetylase isoform that is involved in tumorigenesis.

Non-limiting examples of small molecule inhibitors useful for the invention are presented in Table 2.

Table 2

Small Molecule HDAC Inhibitors [μM] and Their Antitumor Activities *In Vivo*

	Inhibitor	Enzyme IC50 (uM)						% inhibitor of tumor formation in vivo				
Cpd	Structure	HDAC1	HDAC2	HDAC3	HDAC4	HDAC6	H4-Ac	MTT	Cell Cycle Arrest EC	colon	lung	prostate
1		3	25	21	23	>50	1	3	2			
2		3	31	30	35	>30	5	4	8	53 (40,po)	54 (50,ip)	
3		3	22	45	28	>50	5	4	2	55 (40,ip)		

note: for *in vivo* antitumor studies, numbers outside brackets indicate % of inhibition of tumor growth in vivo;
numbers in brackets indicate daily dose of inhibitor used (mg/kg body weight/day);
oral (PO) or intraperitoneal (IP) administration is indicated in brackets.

The reagents according to the invention are useful as analytical tools and as therapeutic tools, including as gene therapy tools. The invention also provides methods and compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects.

In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell comprising contacting the cell with an agent of the first aspect of the invention. By way of non-limiting example, the agent may be an antisense oligonucleotide or a small molecule inhibitor that inhibits the expression of one or more, but less than all, specific histone deacetylase isoforms in the cell.

In one certain embodiment, the invention provides a method comprising contacting a cell with an antisense oligonucleotide that inhibits one or more but less than all histone deacetylase isoforms in the cell. Preferably, cell proliferation is inhibited in the contacted cell. Thus, the antisense oligonucleotides according to the invention are useful in therapeutic approaches to human diseases including benign and malignant neoplasms by inhibiting cell proliferation in cells contacted with the antisense oligonucleotides. The phrase "inhibiting cell proliferation" is used to denote an ability of a histone deacetylase antisense oligonucleotide or a small molecule histone deacetylase inhibitor (or combination thereof) to retard the growth of cells contacted with the oligonucleotide or small molecule inhibitor, as compared to cells not contacted. Such an assessment of cell proliferation can be made by counting contacted and non-contacted cells using a Coulter Cell Counter (Coulter, Miami, FL) or a hemacytometer. Where the cells are in a solid growth (*e.g.*, a solid tumor or organ), such an assessment of cell proliferation can be made by measuring the growth with calipers, and comparing the size of the growth of contacted cells with non-contacted cells. Preferably, the term includes a retardation of cell proliferation that is at least 50% of non-contacted cells. More preferably, the term includes a retardation of cell proliferation that is 100% of non-contacted cells (*i.e.*, the contacted cells do not increase in number or size). Most preferably, the term includes a reduction in the number or size of contacted cells, as compared to non-contacted cells. Thus, a histone deacetylase antisense oligonucleotide or a histone deacetylase small molecule inhibitor that inhibits cell proliferation in a contacted cell may induce the contacted cell to undergo growth retardation, to undergo growth arrest, to undergo programmed cell death (*i.e.*, to apoptose), or to undergo necrotic cell death.

Conversely, the phrase "inducing cell proliferation" and similar terms are used to denote the requirement of the presence or enzymatic activity of a specific histone deacetylase isoform for cell proliferation in a normal (*i.e.*, non-neoplastic) cell. Hence, over-expression of a specific histone deacetylase isoform that induces cell proliferation may or may not lead to increased cell proliferation; however, inhibition of a specific histone deacetylase isoform that induces cell proliferation will lead to inhibition of cell proliferation.

The cell proliferation inhibiting ability of the antisense oligonucleotides according to the invention allows the synchronization of a population of a-synchronously growing cells. For example, the antisense oligonucleotides of the invention may be used to arrest a population of non-neoplastic cells grown *in vitro* in the G1 or G2 phase of the cell cycle. Such synchronization allows, for example, the identification of gene and/or gene products expressed during the G1 or G2 phase of the cell cycle. Such a synchronization of cultured cells may also be useful for testing the efficacy of a new transfection protocol, where transfection efficiency varies and is dependent upon the particular cell cycle phase of the cell to be transfected. Use of the antisense oligonucleotides of the invention allows the synchronization of a population of cells, thereby aiding detection of enhanced transfection efficiency.

The anti-neoplastic utility of the antisense oligonucleotides according to the invention is described in detail elsewhere in this specification.

In yet other preferred embodiments, the cell contacted with a histone deacetylase antisense oligonucleotide is also contacted with a histone deacetylase small molecule inhibitor.

In a few preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide. As mentioned above, the antisense oligonucleotides according to the invention may optionally be formulated well known pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more one or more additional histone deacetylase antisense oligonucleotide(s), and/or one or more histone deacetylase small molecule inhibitor(s), or it may contain any other pharmacologically active agent.

In a particularly preferred embodiment of the invention, the antisense oligonucleotide is in operable association with a histone deacetylase small molecule inhibitor. The term "operable association" includes any association between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor which allows an antisense oligonucleotide to inhibit one or more specific histone deacetylase isoform-encoding nucleic acid expression and allows the histone deacetylase small molecule inhibitor to inhibit specific histone deacetylase isoform enzymatic activity. One or more antisense oligonucleotide of the invention may be operably associated with one or more histone deacetylase small molecule inhibitor. In some preferred embodiments, an antisense oligonucleotide of the invention that targets one particular histone deacetylase isoform (*e.g.*, HDAC-1) is operably associated with a histone deacetylase small molecule inhibitor which targets the same histone deacetylase isoform. A preferred operable association is a hydrolyzable. Preferably, the hydrolyzable association is a covalent linkage between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor. Preferably, such covalent linkage is hydrolyzable by esterases and/or amidases. Examples of such hydrolyzable associations are well known in the art. Phosphate esters are particularly preferred.

In certain preferred embodiments, the covalent linkage may be directly between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor so as to integrate the histone deacetylase small molecule inhibitor into the backbone. Alternatively, the covalent linkage may be through an extended structure and may be formed by covalently linking the antisense oligonucleotide to the histone deacetylase small molecule inhibitor through coupling of both the antisense oligonucleotide and the histone deacetylase small molecule inhibitor to a carrier molecule such as a carbohydrate, a peptide or a lipid or a glycolipid. Other preferred operable associations include lipophilic association, such as formation of a liposome containing an antisense oligonucleotide and the histone deacetylase small molecule inhibitor covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphotidylcholine, cholesterol, phosphatidylethanolamine, and synthetic neoglycolipids, such as syalyllacNAc-HDPE. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when the antisense oligonucleotide is associated with one liposome and the small molecule inhibitor is associated with another liposome.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In one certain embodiment, the agent is an antisense oligonucleotide of the first aspect of the invention, and the method further comprises a pharmaceutically acceptable carrier. The antisense oligonucleotide and the pharmaceutically acceptable carrier are administered for a therapeutically effective period of time. Preferably, the

animal is a mammal, particularly a domesticated mammal. Most preferably, the animal is a human.

The term "neoplastic cell" is used to denote a cell that shows aberrant cell growth. Preferably, the aberrant cell growth of a neoplastic cell is increased cell growth. A neoplastic cell may be a hyperplastic cell, a cell that shows a lack of contact inhibition of growth *in vitro*, a benign tumor cell that is incapable of metastasis *in vivo*, or a cancer cell that is capable of metastases *in vivo* and that may recur after attempted removal. The term "tumorigenesis" is used to denote the induction of cell proliferation that leads to the development of a neoplastic growth.

The terms "therapeutically effective amount" and "therapeutically effective period of time" are used to denote known treatments at dosages and for periods of time effective to reduce neoplastic cell growth. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal, or intrarectal. When administered systemically the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.1 μM to about 10 μM. For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. One of skill in the art will appreciate that such therapeutic effect resulting in a lower effective concentration of the histone deacetylase inhibitor may vary considerably depending on the tissue, organ, or the particular animal or patient to be treated according to the invention.

In a preferred embodiment, the therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μM to about 20 μM. In a particularly preferred embodiment, the therapeutic composition

is administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.05 μM to about 15 μM. In a more preferred embodiment, the blood level of antisense oligonucleotide is from about 0.1 μM to about 10 μM.

For localized administration, much lower concentrations than this may be therapeutically effective. Preferably, a total dosage of antisense oligonucleotide will range from about 0.1 mg to about 200 mg oligonucleotide per kg body weight per day. In a more preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 20 mg oligonucleotide per kg body weight per day. In a most preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 10 mg oligonucleotide per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective amount of a histone deacetylase antisense oligonucleotide is about 5 mg oligonucleotide per kg body weight per day.

In certain preferred embodiments of the third aspect of the invention, the method further comprises administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time. In some preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide, as described *supra*.

The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01μM to about 10μM. In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule

inhibitor from about $0.05\mu M$ to about $10\mu M$. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about $0.1\mu M$ to about $5\mu M$. For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective synergistic amount of histone deacetylase small molecule inhibitor (when administered with an antisense oligonucleotide) is about 5 mg per kg body weight per day.

Certain preferred embodiments of this aspect of the invention result in an improved inhibitory effect, thereby reducing the therapeutically effective concentrations of either or both of the nucleic acid level inhibitor (*i.e.*, antisense oligonucleotide) and the protein level inhibitor (*i.e.*,histone deacetylase small molecule inhibitor) required to obtain a given inhibitory effect as compared to those necessary when either is used individually.

Furthermore, one of skill will appreciate that the therapeutically effective synergistic amount of either the antisense oligonucleotide or the histone deacetylase inhibitor may be lowered or increased by fine tuning and altering the amount of the other component. The invention therefore provides a method to tailor the administration/treatment to the particular exigencies specific to a given animal species or particular patient. Therapeutically effective ranges may be easily determined for example

empirically by starting at relatively low amounts and by step-wise increments with concurrent evaluation of inhibition.

In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell

differentiation comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is an small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time.

In certain embodiments where the agent of the first aspect of the invention is a histone deacetylase small molecule inhibitor, therapeutic compositions of the invention comprising said small molecule inhibitor(s) are administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μM to about

10 μM. In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05 μM to about 10 μM. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about 0.1 μM to about 5 μM. For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day.

In a sixth aspect, the invention provides a method for investigating the role of a particular histone deacetylase isoform in cellular proliferation, including the proliferation of neoplastic cells. In this method, the cell type of interest is contacted with an amount of an antisense oligonucleotide that inhibits the expression of one or more specific histone deacetylase isoform, as described for the first aspect according to the invention, resulting in inhibition of expression of the histone deacetylase isoform(s) in the cell. If the contacted cell with inhibited expression of the histone deacetylase isoform(s) also shows an inhibition in cell proliferation, then the histone deacetylase isoform(s) is required for the induction of cell proliferation. In this scenario, if the contacted cell is a neoplastic cell, and the contacted neoplastic cell shows an inhibition of cell proliferation, then the histone deacetylase isoform whose expression was inhibited is a histone deacetylase isoform that is required for tumorigenesis. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2,

HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

Thus, by identifying a particular histone deacetylase isoform that is required for in the induction of cell proliferation, only that particular histone deacetylase isoform need be targeted with an antisense oligonucleotide to inhibit cell proliferation or induce differentiation. Consequently, a lower therapeutically effective dose of antisense oligonucleotide may be able to effectively inhibit cell proliferation. Moreover, undesirable side effects of inhibiting all histone deacetylase isoforms may be avoided by specifically inhibiting the one (or more) histone deacetylase isoform(s) required for inducing cell proliferation.

As previously indicated, the agent of the first aspect includes, but is not limited to, oligonucleotides and small molecule inhibitors that inhibit the activity of one or more, but less than all, HDAC isoforms. The measurement of the enzymatic activity of a histone deacetylase isoform can be achieved using known methodologies. For example, Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) describe the assessment of histone deacetylase enzymatic activity by the detection of acetylated histones in trichostatin A treated cells. Taunton et al. (*Science* 272: 408-411, 1996) similarly describes methods to measure histone deacetylase enzymatic activity using endogenous and recombinant HDAC. Both Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) and Taunton et al. (*Science* 272: 408-411, 1996) are hereby incorporated by reference.

Preferably, the histone deacetylase small molecule inhibitor(s) of the invention that inhibits a histone deacetylase isoform that is required for induction of cell proliferation is a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of fewer than all histone deacetylase isoforms.

In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8.

The phrase "inducing cell differentiation" and similar terms are used to denote the ability of a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or combination thereof) to induce differentiation in a contacted cell as compared to a cell that is not contacted. Thus, a neoplastic cell, when contacted with a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or both) of the invention, may be induced to differentiate, resulting in the production of a daughter cell that is phylogenetically more advanced than the contacted cell.

In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two of the reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a histone deacetylase small molecule inhibitor, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain preferred embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.

Antisense oligonucleotides that inhibit DNA methyltransferase are described in Szyf and von Hofe, U.S. Patent No. 5,578,716, the entire contents of which are incorporated by reference. DNA methyltransferase small molecule inhibitors include, without limitation, 5-aza-2'-deoxycytidine (5-aza-dC), 5-fluoro-2'-deoxycytidine, 5-aza-cytidine (5-aza-C), or 5,6-dihydro-5-aza-cytidine.

In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In preferred embodiments, the cell proliferation is neoplasia.

For purposes of this aspect, it is unimportant how the specific HDAC isoform is inhibited. The present invention has provided the discovery that specific individual HDACs are involved in cell proliferation or differentiation, whereas others are not. As demonstrated in this specification, this is true regardless of how the particular HDAC isoform(s) is/are inhibited.

By the term "modulating" proliferation or differentiation is meant altering by increasing or decreasing the relative amount of proliferation or differentiation when compared to a control cell not contacted with an agent of the first aspect of the invention. Preferably, there is an increase or decrease of about 10% to 100%. More preferably, there is an increase or decrease of about 25% to 100%. Most preferably, there is an increase or decrease of about 50% to 100%. The term "about" is used herein to indicate a variance of as much as 20% over or below the stated numerical values.

In certain preferred embodiments, the histone deacetylase isoform is selected from HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the appended claims.

EXAMPLES

Example 1
Synthesis and Identification of Antisense Oligonucleotides

Antisense (AS) and mismatch (MM) oligodeoxynucleotides (oligos) were designed to be directed against the 5'- or 3'-untranslated region (UTR) of the targeted gene. Oligos were synthesized with the phosphorothioate backbone and the 4X4 nucleotides 2'-O-methyl modification on an automated synthesizer and purified by preparative reverse-phase HPLC. All oligos used were 20 base pairs in length.

To identify antisense oligodeoxynucleotide (ODN) capable of inhibiting HDAC-1 expression in human cancer cells, eleven phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-1 gene (GenBank Accession No. U50079) were initially screened in T24 cells at 100 nM. Cells were harvested after 24 hours of treatment, and HDAC-1 RNA expression was analyzed by Northern blot analysis. This screen identified HDAC-1 AS1 and AS2 as ODNs with antisense activity to human HDAC-1. HDAC-1 MM oligo was created as a control; compared to the antisense oligo, it has a 6-base mismatch.

Twenty-four phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-2 gene (GenBank Accession No. U31814) were screened as above. HDAC-2 AS was identified as an ODN with antisense activity to human HDAC-2. HDAC-2 MM was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

Twenty-one phosphorothioate ODNs containing sequences complementary to the 5′ or 3′ UTR of the human HDAC-3 gene (GenBank Accession No. AF039703) were screened as above. HDAC-3 AS was identified as an ODN with antisense activity to human HDAC-3. HDAC-3 MM oligonucleotide was created as a control; compared to the antisense oligonucleotide, it contains a 6-base mismatch.

Seventeen phosphorothioate ODNs containing sequences complementary to the 5′ or 3′ UTR of the human HDAC-4 gene (GenBank Accession No. AB006626) were screened as above. HDAC-4 AS1 and AS2 were identified as ODNs with antisense activity to human HDAC-4. HDAC-4 MM1 and MM2 oligonucleotides were created as controls; compared to the antisense oligonucleotides, they each contain a 6-base mismatch.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5′ or 3′ untranslated regions of the human HDAC-5 gene (GenBank Accession No. AF039691) were screened as above. HDAC-5 AS was identified as an ODN with antisense activity to human HDAC-5.

Thirteen phosphorothloate ODNs containing sequences complementary to the 5′ or 3′ untranslated regions of the human HDAC-6 gene (GenBank Accession No. AJ011972) were screened as above. HDAC-6 AS was identified as an ODN with antisense activity to human HDAC-6. HDAC-6 MM oligo was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

Eighteen phosphorothioate ODNs containing sequences complementary to the 5′ or 3′ untranslated regions of the human HDAC-7 gene (GenBank Accession No. AF239243) were screened as above. HDAC-7 AS was identified as an ODN with antisense activity to human HDAC-7.

Fourteen phosphorothioate ODNs containing sequences complementary to the 5′ or 3′ untranslated regions of the human HDAC-8 gene (GenBank Accession No. AF230097) were screened as above. HDAC-8 AS was identified as an ODN with antisense activity to human HDAC-8.

Example 2
HDAC AS ODNs Specifically Inhibit Expression at the mRNA Level

In order to determine whether AS ODN treatment reduced HDAC expression at the mRNA level, human A549 cells were treated with 50 nM of antisense (AS) oligonucleotide directed against human HDAC-3 or its corresponding mismatch (MM) oligo for 48 hours, and A549 cells were treated with 50 nM or 100 nM of AS oligonucleotide directed against human HDAC-1, HDAC-2, HDAC-4, HDAC-5, HDAC-6 or HDAC-7 or the appropriate MM oligonucleotide (100 nM) for 24 hours.

Briefly, human A549 and/or T24 human bladder carcinoma cells were seeded in 10 cm tissue culture dishes one day prior to oligonucleotide treatment. The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were grown under the recommended culture conditions. Before the addition of the oligonucleotides, cells were washed with PBS (phosphate buffered saline). Next, lipofectin transfection reagent (GIBCO BRL Mississauga, Ontario, CA), at a concentration of 6.25 µg/ml, was added to serum free OPTIMEM medium (GIBCO BRL, Rockville, MD), which was then added to the cells. The oligonucleotides to be screened were then added directly to the cells (*i.e.*, one oligonucleotide per plate of cells). Mismatched oligonucleotides were used as controls. The same concentration of oligonucleotide (*e.g.*, 50 nM) was used per plate of cells for each oligonucleotide tested.

Cells were harvested, and total RNAs were analyzed by Northern blot analysis. Briefly, total RNA was extracted using RNeasy miniprep columns (QIAGEN). Ten to twenty μg of total RNA was run on a formaldehyde-containing 1% agarose gel with 0.5 M sodium phosphate (pH 7.0) as the buffer system. RNAs were then transferred to nitrocellulose membranes and hybridized with the indicated radiolabeled DNA probes. Autoradiography was performed using conventional procedures.

Figures 9A-9I present results of experiments conducted with HDAC-1 (Figure 9A), HDAC-2 (Figure 9B), HDAC-6 (Figure 9C), HDAC-3 (Figure 9D), HDAC-4 (Figures 9E and 9F), HDAC-5 (Figure 9G), HDAC-7 (Figure 9H), and HDAC-8 (Figure 9I) AS ODNs.

Treatment of cells with the respective HDAC AS ODN significantly inhibits the expression of the targeted HDAC mRNA in human A549 cells.

Example 3
HDAC OSDNs Inhibit HDAC Protein Expression

In order to determine whether treatment with HDAC OSDNs would inhibit HDAC protein expression, human A549 cancer cells were treated with 50 nM of paired antisense or its mismatch oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 48 hours. OSDN treatment conditions were as previously described.

Cells were lysed in buffer containing 1% Triton X- 100, 0.5 % sodium deoxycholate, 5 mM EDTA, 25 mM Tris-HC1, pH 7.5, plus protease inhibitors. Total protein was quantified by the protein assay reagent from Bio-Rad (Hercules, CA). 100 ug of total protein was analyzed by SDS-PAGE. Next, total protein was transferred onto a PVDF membrane and probed with various HDAC-specific primary antibodies. Rabbit anti-

HDAC-1 (H-51), anti-HDAC-2 (H-54) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1:500 dilution. Rabbit anti-HDAC-3 antibody (Sigma, St. Louis, MO) was used at a dilution of 1:1000. Anti-HDAC-4 antibody was prepared as previously described (Wang, S.H. *et al.*, (1999) *Mol. Cell. Biol.* **19**:7816-27), and was used at a dilution of 1:1000. Anti-HDAC-6 antibody was raised by immunizing rabbits with a GST fusion protein containing a fragment of HDAC-6 protein (amino acid #990 to #1216, GenBank Accession No. AAD29048). Rabbit antiserum was tested and found only to react specifically to the human HDAC-6 isoform. HDAC-6 antiserum was used at 1:500 dilution in Western blots to detect HDAC-6 in total cell lysates. Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ).

As shown in Figure 10A, the treatment of cells with HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 ODNs for 48 hours specifically inhibits the expression of the respective HDAC isotype protein. Figure 10B presents dose dependent response for the inhibited expression of HDAC-1 protein in cells treated with two HDAC-1 AS ODNs. As predicted, treatment of cells with the respective mismatch (MM) control oligonucleotide does not result in a significant decrease in HDAC-1 protein expression in the treated cells.

In order to demonstrate that the level of HDAC protein expression is an important factor in the cancer cell phenotype, experiments were done to determine the level of HDAC isotype expression in normal and cancer cells. Western blot analysis was performed as described above.

The results are presented in Table 3 clearly demonstrate that HDAC-1, HDAC-2, HDAC-3, HDAC-4, and HDAC-6, isotype proteins are overexpressed in cancer cell lines.

Table 3

Expression Level of HDAC Isotypes in Human Normal and Cancer Cells

States of Cell	Tissue Type	Cell Designation	HDAC-1	HDAC-2	HDAC-3	HDAC-4	HDAC-6
Normal	Breast Epithelial	HMEC	-	+	++	+	+
Normal	Foreskin Fibroblasts	MRHF	-	+	+	++	+
Cancer	Bladder	T24	+++	++	+++	++	+++
Cancer	Lung	A549	++	+++	+++	+++	++
Cancer	Colon	SW48	+++	+++	+++	+++	+++
Cancer	Colon	HCT116	++++	+++	+++	++++	+++
Cancer	Colon	HT29	+++	+++	+++	+++	+++
Cancer	Colon	NCI-H446	++	++++	+++	++++	++
Cancer	Cervix	Hela	+++	++++	+++	+++	+++
Cancer	Prostate	DU145	+++	+++	+++	++++	+++
Cancer	Breast	MDA-MB-231	++	+++	+++	+++	++++
Cancer	Breast	MCF-7	+++	+++	+++	++	++
Cancer	Breast	T47D	+++	+++	+++	++	+++
Cancer	Kidney	293T	+++	++++	++++	++	++
Cancer	Leukemia	K562	+++	++++	++++	++++	++++
Cander	Leukemia	Jurkat T	+++	++	++++	++	++

(-): not detectable; (+): detectable; (++): 2X over (+); (+++): 5X over (+); (++++): 10X over (+)

Example 4

Effect of HDAC Isotype Specific OSDNs on Cell Growth and Apoptosis

In order to determine the effect of HDAC OSDNs on cell growth and cell death through apoptosis, A549 or T24 cells, MDAmb231 cells, and HMEC cells (ATCC, Manassas, VA) were treated with HDAC OSDNs as previously described.

For the apoptosis study, cells were analyzed using the Cell Death Detection ELISA ^{Plus} kit (Roche Diagnostic GmBH, Mannheim, Germany) according to the manufacturer's directions. Typically, 10,000 cells were plated in 96-well tissue culture dishes for 2 hours before harvest and lysis. Each sample was analyzed in duplicate. ELISA reading was done using a MR700 plate reader (DYNEX Technology, Ashford, Middlesex, England) at 410 nm. The reference was set at 490 nm.

For the cell growth analysis, human cancer or normal cells were treated with 50 nM of paired AS or MM oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 72 hours. Cells were harvested and cell numbers counted by trypan blue exclusion using a hemocytometer. Percentage of inhibition was calculated as (100 - AS cell numbers/control cell numbers)%.

Results of the study are shown in Figures 11-13, and in Table 4 and Table 5. Treatment of human cancer cells by HDAC-4 AS, and to a lesser extent, HDAC 1 AS, induces growth arrest and apoptosis of various human cancer. The corresponding mismatches have no effect. The effects of HDAC-4 AS or HDAC-1 AS on growth inhibition and apoptosis are significantly reduced in human normal cells. In contrast to the effects of HDAC-4 or HDAC-1 AS oligos, treatment with human HDAC-3 and HDAC-6 OSDNs has no effect on cancer cell growth or apoptosis, and

treatment with human HDAC-2 OSDN has a minimal effect on cancer cell growth inhibition. Since T24 cells are p53 null and A549 cells have functional p53 protein, this induction of apoptosis is independent of p53 activity.

Table 4

Effect of HDAC Isotype-Specific OSDNs on Human Normal and Cancer Cells Growth Inhibition (AS vs. MM)

	Cancer Cells	Normal Cells		
	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	++(+)	+(+)	+/-	+/-
HDAC-2 AS	+(+)	+/-	-	+/-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	++	++	+/-
HDAC-6 AS	-	-	+/-	-

"-": no inhibition, "+": <50% inhibition, "++": 50-75% inhibition, "+++": >75% inhibition

Table 5

Effect of HDAC Isotype-Specific OSDNs on Human Normal and Cancer Cells Apoptosis After 48 Hour Treatment

	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	+	-		-
HDAC-2 AS	-	-	-	-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	+	++	-
HDAC-6 AS	-	-	-	-
TSA (100ng/ml)	++	++	++	+

"-": < = 2x fold over non-specific background; "+": 2-3X fold; "++": 3-5X fold; "+++": 5-8X fold; "++++": 8X fold

Example 5

Inhibition of HDAC Isotypes Induces the Expression of Growth Regulatory Genes

In order to understand the mechanism of growth arrest and apoptosis of cancer cells induced by HDAC-1 or HDAC-4 AS treatment, RNase protection assays were used to analyze the mRNA expression of cell growth regulators (p21 and *GADD45*) and proapoptotic gene *Bax*.

Briefly, human cancer A549 or T24 cells were treated with HDAC isotype-specific antisense oligonucleotides (each 50 nM) for 48 hours. Total RNAs were extracted and RNase protection assays were performed to analyzed the mRNA expression level of p21 and *GADD45*. As a control, A549 cells were treated by lipofectin with or without TSA (250 ng/ml) treatment for 16 hours. These RNase protection assays were done

according to the following procedure. Total RNA from cells was prepared using "RNeasy miniprep kit" from QIAGEN following the manufacturer's manual. Labeled probes used in the protection assays were synthesized using "hStress-1 multiple-probe template sets" from Pharmingen (San Diego, California, U.S.A.) according to the manufacturer's instructions. Protection procedures were performed using "RPA II™ Ribonuclease Protection Assay Kit" from Ambion, (Austin, Tx) following the manufacturer's instructions. Quantitation of the bands from autoradiograms was done by using Cyclone™ Phosphor System (Packard Instruments Co. Inc., Meriden, CT). The results are shown in Figures 14, 15 and Table 6.

Table 6

Up-Regulation of p21, *GADD45* and *Bax* After Cell Treatment with Human HDAC Isotype-Specific Antisenses

	A549			T24		
	p21	*GADD45*	*Bax*	p21	*GADD45*	*Bax*
HDAC-1	1.7	5.0	0.8	2.4	3.4	0.9
HDAC-2	1.1	1.2	1.0	1.0	1.0	0.9
HDAC-3	0.7	0.9	1.0	0.9	1.0	1.0
HDAC-4	3.1	5.7	2.6	2.8	2.7	1.9
HDAC-6	1.0	1.0	1.0	1.0	0.8	1.1
TSA vs lipofectin	2.8	0.6	0.8			

Values indicate the fold induction of transcription as measured by RNase protection analysis for the respective AS vs. MM HDAC isotype-specific oligos.

Results of the experiments are presented in Table 6. The inhibition of HDAC-4 in both A549 and T24 cancer cells dramatically up-regulates both p21 and *GADD45* expression. Inhibition of HDAC-1 by antisense oligonucleotides induces p21 expression but more greatly induces *GADD45* expression. Inhibition of HDAC-4, upregulates *Bax* expression in both A549 and T24 cells. The effect of HDAC-4 AS treatment (50 nM, 48 hrs) on p21 induction in A549 cells is comparable to that of TSA (0.3 to 0.8 uM, 16 hrs).

Experiments were also conducted to examine the affect of HDAC antisense oligonucleotides on HDAC protein expression. In A549 cells, treatment with HDAC-4 antisene oligonucleotides results in a dramatic increase in the level of p21 protein (Figure 15).

Example 6

Cyclin Gene Expression Is Repressed by HDAC-1 AS Treatment

Human cancer A549 cells were treated with AS1, AS2 or MM oligo directed human HDAC1 for 48 hours. Total cell lysates were harvested and analyzed by Western blot using antibodies against human HDAC1, cyclin B1, cyclin A and actin (all from Santa Cruz Biotechnology, Inc., Santa Cruz, California). AS1 or AS2 both repress expression of cyclin B1 and A. Downregulation of cyclin A and B1 expression by AS1 and AS2 correlates well with their ability to inhibit cancer cell growth. (Figure 16)

Example 7

Inhibition of Growth in Soft Agar

1.3 g granulated agar (DIDFCO) was added to 100 ml deionized water and boiled in a microwave to sterilize. The boiled agar was held at 55•C until further use. Iscove's Modified Dulbecco's Medium (GIBCO/BRL), 100x Penicillin-Streptomycin-Glutamine (GIBCO/BRL) and fetal bovine serum (medicorp) were pre-warmed at 37•C. To 50 ml sterile tubes was added 9 ml Isove's medium, 2 ml fetal bovine serum and 0.2 ml 100x Pen-Strep-Gln. Then 9 ml 55•C 1.3% agar was added to each tube. The tube contents were mixed immediately, avoiding air bubbles, and 2.5 ml of the mixture was poured into each sterile 6 cm petri dish to form a polymerized bottom layer. Dishes with polymerized bottom layers were then put in a CO2 incubator at 37•C until further use. In 50 ml sterile tubes were prewarmed at 37•C for each 4 cell lines/samples, 20 ml Iscove's medium, 0.4 ml 100x Pen-Strp-Gln and 8 ml fetal bovine serum. Cells were trypsinized and counted by trypan blue staining and 20,000 cells were aliquotted into a sterile 15 ml tube. To the tube was then added DMEM with low glucose (GIBCO/BRL) + 10% fetal bovine serum + Pen-Strep-Gln to a final volume of 1 ml. To the prewarmed 37•C mix in the 50 ml tube was quickly added 8 ml 55•C 1.3% agar, which was then mixed well. Nine ml of this mixture was then aliquotted to each 1 ml cells in the 15 ml tube which is then mixed and 5 ml aliquotted onto the ploymerized bottom layer of the 6 cm culture plates and allowed to polymerize at room temperature. After polymerization, 2.5 ml bottom layer mix was gently added over the cell layer. Plates were wrapped up in foil paper and

incubated in a CO2 incubator at 37°•C for three weeks, at which time colonies in agar are counted. The results are shown in Figure 17.

These results demonstrate that an antisense oligonucleotide complementary to HDAC-1 inhibits growth of A549 cells in soft agar, but antisense oligonucleotides complementary to HDAC-2 or HDAC-6, or mismatch controls, do not.

Example 8

Inhibition of HDAC Isotypes by Small Molecules

In order to demonstrate the identification of HDAC small molecule inhibitors, HDAC small molecule inhibitors were screened in histone deacetylase enzyme assays using various human histone deacetylase isotypic enzymes (*i.e.*, HDAC-1, HDAC-3, HDAC-4 and HDAC-6). Cloned recombinant human HDAC-1, HDAC-3 and HDAC-6 enzymes, which were tagged with the Flag epitope (Grozinger, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 96:4868-4873 (1999)) in their C-termini, were produced by a baculovirus expression system in insect cells.

Flag-tagged human HDAC-4 enzyme was produced in human embronic kidney 293 cells after transformation by the calcium phosphate precipitation method. Briefly, 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and antibiotics. Plasmid DNA encoding Flag-tagged human HDAC-4 was precipitated by ethanol and resuspend in sterile water. DNA-calcium precipitates, formed by mixing DNA, calcium choloride and 2XHEPES-buffered saline solution, were left on 293 cells for 12-16 hours. Cells were return to serum-contained DMEM medium and harvested at 48 hour post transfection for purification of Flag-tagged HDAC-4 enzyme.

HDAC-1 and HDAC-6 were purified on a Q-Sepharose column, followed by an anti-Flag epitope affinity column. The other HDAC isotypes, HDAC-3 and HDAC-4, were purified directly on an anti-Flag affinity column.

For the deacetylase assay, 20,000 cpm of an [^{3}H]-metabolically-labeled acetylated histone was used as a substrate. Histones were incubated with cloned recombinant human HDAC enzymes at 37^{0}C. For the HDAC-1 asasy, the incubation time was 10 minutes, and for the HDAC-3, HDAC-4 and HDAC-6 assays, the incubation time was 2 hours. All assay conditions were pre-determined to be certain that each reaction was linear. Reactions were stopped by adding acetic acid (0.04 M, final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate, and the released [^{3}H]-acetic acid was quantified by liquid scintillation counting. For the inhibition studies, HDAC enzyme was preincubated with test compounds for 30 minutes at 4^{0}C prior to the start of the enzymatic assay. IC_{50} values for HDAC enzyme inhibitors were identified with dose response curves for each individual compound and, thereby, obtaining a value for the concentration of inhibitor that produced fifty percent of the maximal inhibition.

Example 9

Inhibition of HDAC Activity in Whole Cells by Small Molecules

T24 human bladder cancer cells (ATCC, Manassas, VA) growing in culture were incubated with test compounds for 16 hours. Histones were extracted from the cells by standard procedures (see *e.g.* Yoshida *et al.*, *supra*) after the culture period. Twenty μg total core histone protein was loaded onto SDS/PAGE and transferred to nitrocellulose membranes, which were then reacted with polyclonal antibody specific for acetylated histone H-4 (Upstate Biotech Inc., Lake Placid, NY). Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000

to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ). After exposure to film, acetylated H-4 signal was quantitated by densitometry.

The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit histone deacetylation in whole cells.

Example 10

Inhibition of Cancer Cell Growth by HDAC Small Molecule Inhibitors

Two thousand (2,000) human colon cancer HCT116 cells (ATCC, Manassas, VA) were used in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to quantitatively determine cell proliferation and cytotoxicity. Typically, HCT116 cells were plated into each well of the 96-well tissue culture plate and left overnight to attach to the plate. Compounds at various concentrations were added into the culture media (final DMSO concentration 1%) and incubated for 72 hours. MTT solution (obtained from Sigma as powder) was added and incubated with the cells for 4 hours at 37°C in incubator with 5% CO_2. During the incubation, viable cells convert MTT to a water-insoluble formazan dye. Solubilizing buffer (50% N,N-dimethylformamide, 20% SDS, pH 4.7) was added to cells and incubated for overnight at 37C in incubator with 5% CO_2. Solubilized dye was quantitated by colorimetric reading at 570 nM using a reference of 630 nM. Optical density values were converted to cell number values by comparison to a standard growth curve for each cell line. The concentration test compound that reduces the total cell number to 50% that of the control treatment, *i.e.*, 1% DMSO, is taken as the EC_{50} value.

The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can affect cell proliferation.

Example 11

Inhibition by Small Molecules of Tumor Growth in a Mouse Model

Female BALB/c nude mice were obtained from Charles River Laboratories (Charles River, NY) and used at age 8-10 weeks. Human prostate tumor cells (DU145, 2 x 10^6) or human colon cancer cells (HCT116; 2 x 10^6) or small lung core A549 2 x 10^6 were injected subcutaneously in the animal's flank and allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times, then approximately 30 mg tumor fragments were implanted subcutaneously through a small surgical incision under general anaesthesia. Small molecule inhibitor administration by intraperotineal or oral administration was initiated when the tumors reached a volume of 100 mm^3. For intraperotineal administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/day) were dissolved in 100% DMSO and administered daily intraperitoneally by injection. For oral administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/days) were dissolved in a solution containing 65% polyethylene glycol 400 (PEG 400 (Sigma-Aldridge, Mississauga, Ontario, CA, Catalogue No. P-3265), 5% ethanol, and 30% water. Tumor volumes were monitored twice weekly up to 20 days. Each experimental group contained at least 6-8 animals. Percentage inhibition was calculated using volume of tumor from vehicle-treated mice as controls.

The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit the growth of tumor cells *in vivo*.

Example 12

Upregulation of p21 Expression and Down regulation of Cyclin Gene Expression Following Treatment with Small Molecule Inhibitor

Sulfonamide aniline (compound 3, Table 2) is a small molecule HDAC1 specific inhibitor. Human HCT116 cells were treated with escalating doses of compound 3 for 16 hours. Total cell lysates were harvested and expression of p21 ^{WAF1}, cyclin B1, cyclin A and actin was analyzed by Western blot. Ariti-p21 ^{WAF1} antibody was purchased from BD Transduction Laboratories (BD Pharmingen Canada, Missasagua, Ontario). Compound 3 clearty upregulates expression of p21 ^{WAF1} and represses the expression of cyclin A and B1. The expression profile of these cell cycle regulators correlates well with the ability of compound 3 to inhibit HCT116 proliferation in MTT assays (see Table 2),

Example 13

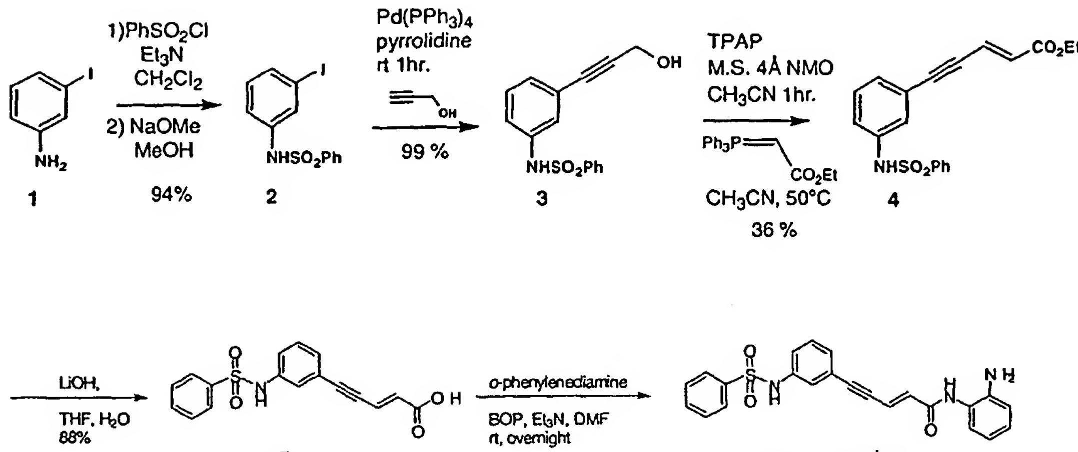
Cell Cycle Arrest Induced by HDAC Small Molecule Inhibtiors

Human cancer HCT116 cells were plated at 2X10^5 per 10-cm dish and were left to attach to the dish overnight in the incubator. Cells were treated with small molecule inhibitors at various concentrations (1 uM and 10 uM, typically, dissolved in DMSO) for 16 hours. Cells were harvested by trypsinization and washed once in 1X PBS (phosphate buffered saline). The cells were resuspended in about 200ul 1X PBS and were fixed by slowly adding 1 ml 70% ethanol at –20° C and were left at least overnight at

–20° C. Fixed cells were centrifuged at low speed (1,000 rpm) for 5 minutes, and the cell pellets were washed again with 1X PBS. Nucleic acids from fixed cells were incubated in a staining solution (0.1% (w/v) glucose in 1X PBS containing 50 ug/ml propidium iodide) (Sigma-Aldridge, Mississauga, Ontario, CA) and RNase A (final 100 units/ml, (Sigma-Aldridge, Mississauga, Ontario, CA) for at least 30 minutes in the dark at 25° C. DNA content was measured by using a fluorescence-activated cell sorter (FACS) machine. Treatment of cells with all HDAC small molecule inhibitors in Table 2 results in a significant accumulation of cancer cell in G2/M phase of the cell cycle and concomitantly reduce the accumulation of cancer cells in S phase of the cell cycle. The ratio of cells in G2/M phase vs. cells in the S phase was determined. The Effective concentration (EC) of a small molecule inhibitor to induce a (G2+M)/S ratio of 2.5 is calculated, as shown in Table 2.

Example: 14
Synthesis of Small Molecule Compound No. 2

The following provides a synthesis scheme for small molecule Compound No. 2 from Table 2.



Compound 2

Step 1: 3-(benzenesulfonylamino)-phenyl iodide (2)

To a solution of 3-iodoaniline (5 g, 22.8 mmol), in CH_2Cl_2 (100 mL), were added at room temperature Et_3N (6.97 mL) followed by benzenesulfonyl chloride (5.84 mL). The mixture was stirred 4 h then a white precipitate was formed. A saturated aqueous solution of $NaHCO_3$ was added and the phases were separated. The aqueous layer was extracted several times with CH_2Cl_2 and the combined extracts were dried over ($MgSO_4$) then evaporated. The crude mixture was dissolved in MeOH (100 mL) and NaOMe (6 g), was added and the mixture was heated 1 h at 60 °C. The solution became clear with time and HCl (1N) was added. The solvent was evaporated under reduced pressure then the aqueous phase was extracted several times with CH_2Cl_2. The combined organic extracts were dried over ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography using (100% CH_2Cl_2) as solvent yielding the title compound **21** (7.68g, 94 %) as yellow solid.

^{1}H NMR: (300 MHz, $CDCl_3$): δ 7.82-7.78 (m, 2H), 7.60-7.55 (m, 1H), 7.50-7.42 (m, 4H), 7.10-7.06 (m, 1H), 6.96 (t, J = 8Hz, 1H), 6.87 (broad s, 1H).

Step 2: 3-(benzenesulfonylamino)-phenyl-propargylic alcohol (3)

To a solution of 2 (500 mg, 1.39 mmol) in pyrrolidine (5 mL) at room temperature was added $Pd(PPh_3)_4$ (80 mg, 0.069 mmol), followed by CuI (26 mg, 0.139 mmol). The mixture was stirred until complete dissolution. Propargylic alcohol (162 •L, 2.78 mmol) was added and stirred 6 h at room temperature. Then the solution was treated with a saturated aqueous solution of NH_4Cl and extracted several times with AcOEt. The combined organic extracts were dried over ($MgSO_4$) then evaporated. The residue was purified by flash chromatography using hexane/AcOEt (1:1) as solvent mixture yielding 3 (395 mg, 99 %) as yellow solid.

^{1}H NMR: (300 MHz, $CDCl_3$): δ 7.79-7.76 (m, 2H), 7.55-7.52 (m,1H), 7.45 (t, J = 8Hz, 2H), 7.19-7.15 (m, 3H), 7.07-7.03 (m, 1H), 4.47 (s, 2H).

Step 3: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoate (4)

To a solution of 3 (2.75 g, 9.58 mmol) in CH_3CN (150 mL) at room temperature were added 4-methylmorpholine N-oxide (NMO, 1.68 g, 14.37 mmol) followed by tetrapropylammonium perruthenate (TPAP, 336 mg, .958 mmol). The mixture was stirred at room temperature 3 h, and then filtrated through a Celite pad with a fritted glass funnel. To the filtrate carbethoxymethylenetriphenyl-phosphorane (6.66 g, 19.16 mmol) was added and the resulting solution was stirred 3 h at room temperature. The solvent was evaporated and the residue was dissolved in CH_2Cl_2 and washed with a saturated aqueous solution of NH_4Cl. The aqueous layer was extracted several times with CH_2Cl_2 then the combined organic extract were dried over ($MgSO_4$) and evaporated. The crude material was purified

by flash chromatography using hexane/AcOEt (1:1) as solvent mixture giving **4** (1.21 g, 36%) as yellow oil.

^{1}H NMR: (300 MHz, $CDCl_3$): δ 7.81 (d, J = 8Hz, 2H), 7.56-7.43 (m, 3H), 7.26-7.21 (m, 3H), 7.13-7.11 (m, 1H), 6.93 (d, J = 16 Hz, 1H), 6.29 (d, J = 16Hz, 1H), 4.24 (q, J = 7 Hz, 2H), 1.31 (t, J = 7Hz, 3H).

Step 4: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenic acid (5)

To a solution of **4** (888 mg, 2.50 mmol) in a solvent mixture of THF (10 mL) and water (10 mL) at room temperature was added LiOH (1.04 g, 25.01 mmol). The resulting mixture was heated 2 h at 60 °C and treated with HCl (1N) until pH 2. The phases were separated and the aqueous layer was extracted several times with AcOEt. The combined organic extracts were dried over ($MgSO_4$) then evaporated. The crude residue was purified by flash chromatography using CH_2Cl_2/MeOH (9:1) as solvent mixture yielding **5** (712 mg, 88 %), as white solid.

^{1}H NMR: (300 MHz, DMSO-d_6): δ 7.78-7.76 (m, 2H), 7.75-7.53 (m, 3H), 7.33-7.27 (m, 1H), 7.19-7.16 (m, 3H), 6.89 (d, J = 16 Hz, 1H), 6.33 (d, J = 16 Hz, 1H).

Step 5: **Compound 2**

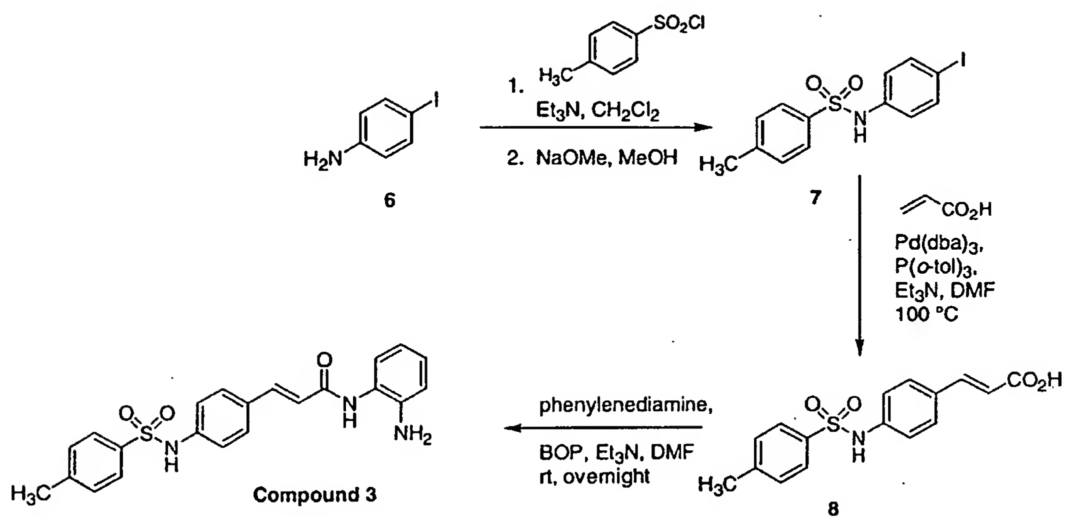
Coupling of **5** with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) afforded the anilide **Compound 2**.

^{1}H NMR: (300 MHz, DMSO d_6): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

^{13}C NMR: (75 MHz, DMSO d_6): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 15
Synthesis of Small Molecule Compound No. 3

The following provides a synthesis scheme for Compound No. 3 from Table 2.



Step 1: 3-[4-(toluenesulfonylamino)-phenyl]-2-propenoic acid (8)

To a solution of 7 (1.39 mmol), in DMF (10 mL) at room temperature were added tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$; 1.67 mmol), tri-*o*-tolylphosphine ($P(o\text{-tol})_3$, 0.83 mmol), Et_3N (3.48 mmol) and finally acrylic acid (1.67 mmol). The resulting solution was degassed and purged several times with N_2 then heated overnight at 100 °C. The solution was filtrated through a Celite pad with a fritted glass funnel then the filtrate was evaporated. The residue was purified by flash chromatography using CH_2Cl_2/MeOH (95:5) as solvent mixture yielding the title compound 8.

Step 2: N-Hydroxy-3-[4-(benzenesulfonylamino)-phenyl]-2-propenamide

(Compound 3)

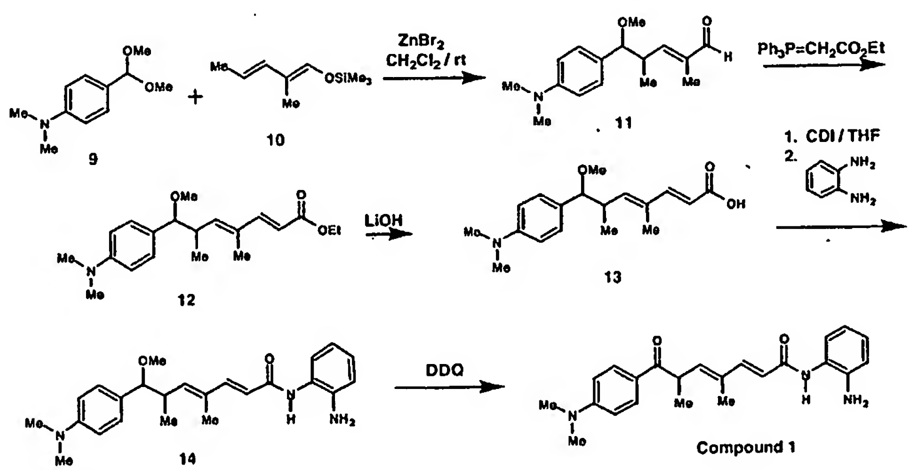
The acid 8 was coupled with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) to afford the anilide **Compound 3.**

^{1}H NMR: (300 MHz, DMSO d_6): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

^{13}C NMR: (75 MHz, DMSO d_6): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 16
Synthesis of Small Molecule No. Compound 1

The following provides a synthesis scheme for small molecule Compound No. 1 from Table 2.



Step 1: **(11)**

To a stirred solution of *p*-anisaldehyde dimethyl acetal **(9)** (10 mmol) in dry CH_2Cl_2 (60 mL) at rt was added 2-methyl-1-trimethylsilyloxypenta-1,3-diene **(10)** (*Tetrahedron*, **39**: 881 (1983)) (10 mmol) followed by catalytic amount of anhydrous $ZnBr_2$ (25 mg). After being stirred for 5 h at rt, the reaction was quenched with water (20 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. Purification of the crude product by flash silica gel chromatography (25% ethyl acetate in hexane) afforded the desired aldehyde **11** in 68% yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer**: ^{1}H NMR (300 MHz, $CDCl_3$) • 9.29 (s, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.67 (d, J =8.4 Hz, 2H), 6.29 (dq, J = 9.9, 1.2 Hz, 1H), 3.96 (d, J = 6.6 Hz, 1H), 3.20 (s, 3H), 3.05 (m, 1H), 2.94 (s, 6H), 1.60 (d, J = 0.9 Hz, 3H), 1.12 (d, J = 6.9 Hz, 3H).

Step 2: **(12)**

A mixture of aldehyde **11** (5.14 mmol) and ethyl (triphenyl-phosphoranylidene)acetate (2.15 g, 6.16 mmol) in toluene (25 mL) was heated at reflux overnight under N_2. After removal of the solvent under reduced pressure, the crude product obtained was purified by flash silica gel chromatography (10% ethyl acetate in hexane) to give the title compound **12** in 96 % yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21 (dd, J = 15.6, 0.9 Hz, 1H), 7.06 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 5.69 (d, J = 15.6Hz, 1H), 5.67 (br. d, J = 9.0 Hz, 1H), 4.17 (q, J = 7.2 Hz, 2H), 3.87 (d, J = 6.9Hz, 1H), 3.18 (s, 3H), 2.93 (s, 6H), 2.81 (m, 1H), 1.59 (d, J = 1.2 Hz, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.05 (d, 6.6 Hz, 3H).

Step 3: (13)

To a stirred solution of diene ester **12** (1.24 mmol) in methanol (10 mL) at rt was added aqueous LiOH 0.5 N solution (1.7mmol). After being stirred at 40 °C for 16 h, methanol was removed under reduced pressure and the resulting aqueous solution was acidified with 3*N* HCl (pH = ca. 4), extracted with ethyl acetate (25 × 3 mL), dried ($MgSO_4$), and concentrated under reduced pressure to give the desired carboxylic acid **13** in 98 % yield: **major isomer**: ^1H NMR (300 MHz, CD_3OD) δ 7.21 (d, J = 15.6, 0.6 Hz, 1H), 7.04 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H), 5.61 (d, J = 15.6 Hz, 1H), 5.60 (br. d, J =10.0 Hz, 1H), 3.85 (d, J = 7.5 Hz, 1H), 3.13 (s, 3H), 2.87 (s, 6H), 2.81 (m, 1H), 1.52 (d, J = 1.5 Hz, 3H), 1.06 (d, J = 6.6 Hz, 3H).

Step 4: (14)

To a solution of carboxylic acid **13** (0.753 mmol) in anhydrous THF (10 mL) was added 1,1'-carbonyldiimidazole (0.790 mmol) at rt, and the mixture was stirred overnight. To the resulting solution was added 1,2-phenylenediamine (5.27 mmol), followed by trifluoroacetic acid (52 μl), and the reaction mixture was stirred for 16 h at rt. The reaction mixture was diluted with ethyl acetate (30 mL), washed with saturated $NaHCO_3$ solution (5 mL) and then water (10 mL), dried ($MgSO_4$), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in toluene) afforded the title compound **14** in 61% yield, as a mixture of two isomers in a ca.3 : 1 ratio: **major isomer**: ^1H NMR (300 MHz, CD_3OD) δ 7.28-7.02 (m, 5H), 6.79 (m, 2H), 6.68 (d, J = 8.7 Hz, 2H), 5.83 (d, J = 15.0 Hz, 1H), 5.69 (d, J = 9.6 Hz, 1H), 3.87 (d, J = 6.9 Hz, 1H), 3.19 (s, 3H), 2.94 (s, 6H), 2.80 (m, 1H), 1.61 (br. s, 3H), 1.07 (d, J = 6.6 Hz, 3H).

Step 5: (Compound 1)

To a stirred solution of compound **14** (0.216 mmol) in wet benzene (2 mL, benzene : H_2O = 9 : 1) at room temperature was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.432 mmol). After being stirred vigorously for 15 min., the mixture was diluted with ethyl acetate (30 mL), washed with water (2 × 5 mL), dried (anhydr.$MgSO_4$), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in hexanes, and then ethyl acetate only) afforded the title compound 35 (6 mg, 7% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 (d, J = 9.0, 2H), 7.87 (br. s, 1H), 7.29 (d, J = 15.6 Hz, 1H), 7.27 (d, 7.8 Hz, 1H), 7.00 (m, 1H), 6.72 (m, 2H), 6.62 (d, J = 9.0 Hz, 2H), 5.97 (d, J = 15.6 Hz, 1H), 5.97 (d, J = 9.3Hz, 1H), 4.34 (dq, J = 9.3, 6.9 Hz, 1H), 3.03 (s, 3H), 1.87 (br. s, 3H), 1.29 (d, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$)
δ 12.6, 17.6, 39.9, 40.8, 110.7, 118.0, 119.0, 119.3, 123.8, 124.4, 125.1, 126.9, 130.6, 132.5, 140.8, 146.2, 153.4, 164.8, 198.6.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodimemts of the invention described herein. Such equivalents are intended to be encompasssed by the following claims.

What is claimed is:

1. An agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms.

2. The agent according to claim 1, wherein the agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms, is an oligonucleotide.

3. The oligonucletide according to claim 2, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA that encodes a portion of one or more histone deacetylase isoforms.

4. The oligonucleotide according to claim 3, wherein the oligonucleotide is a chimeric oligonucleotide.

5. The oligonucleotide according to claim 3, wherein the oligonucleotide is a hybrid oligonucleotide.

6. The oligonucleotide according to claim 3, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA selected from the group consisting of

(a) a nucleic acid molecule encoding a portion of HDAC-1 (SEQ ID NO:2),

(b) a nucleic acid molecule encoding a portion of HDAC-2 (SEQ ID NO:4),

(c) a nucleic acid molecule encoding a portion of HDAC-3 (SEQ ID NO:6),

(d) a nucleic acid molecule encoding a portion of HDAC-4 (SEQ ID NO:8),

(e) a nucleic acid molecule encoding a portion of HDAC-5 (SEQ ID NO:10),

(f) a nucleic acid molecule encoding a portion of HDAC-6 (SEQ ID NO:12),

(g) a nucleic acid molecule encoding a portion of HDAC-7 (SEQ ID NO:14), and

(h) a nucleic acid molecule encoding a portion of HDAC-8 (SEQ ID NO:18).

7. The oligonucleotide according to claim 6 having a nucleotide sequence of from about 13 to about 35 nucleotides.

8. The oligonucleotide according to claim 6 having a nucleotide sequence of from about 15 to about 26 nucleotides.

9. The oligonucleotide according to claim 6 having one or more phosphorothioate internucleoside linkage, being 20-26 nucleotides in length, and being modified such that the terminal four nucleotides at the 5′ end of the oligonucleotide and the terminal four nucleotides at the 3′ end of the oligonucleotide each have 2′-O- methyl groups attached to their sugar residues.

10. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-1 (SEQ ID NO:2).

11. The oligonucleotide according to claim 10 that is SEQ ID NO:17 or SEQ ID NO:18.

12. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-2 (SEQ ID NO:4).

13. The oligonucleotide according to claim 12 that is SEQ ID NO:20.

14. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-3 (SEQ ID NO:6).

15. The oligonucleotide according to claim 14 that is SEQ ID NO:22.

16. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-4 (SEQ ID NO:8).

24. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-8 (SEQ ID NO:16).

25. The oligonucleotide according to claim 24 that is SEQ ID NO:32 or SEQ ID NO:33.

26. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the agent according to claim 1.

27. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the oligonucleotide according to claim 3.

28. The method according to claim 27, wherein cell proliferation is inhibited in the contacted cell.

29. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth retardation.

30. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth arrest.

31. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo programmed cell death.

32. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo necrotic cell death.

33. The method according to claim 27, further comprising contacting the cell with a histone deacetylase small molecule inhibitor.

34. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the agent of claim 1.

35. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the oligonucleotide of claim 3.

36. The method according to claim 35, wherein the animal is a human.

37. The method according to claim 35, further comprising administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time.

38. A method for identifying a histone deacetylase isoform that is required for the induction of cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in the induction of cell proliferation indicates that the histone deacetylase isoform is required for the induction of cell proliferation.

39. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

40. A method for identifying a histone deacetylase isoform that is required for cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in cell proliferation indicates that the histone deacetylase isoform is required for cell proliferation.

41. The method according to claim 40, wherein the inhibitory agent is an oligonucleotide of claim 3.

42. A method for identifying a histone deacetylase isoform that is required for the induction of cell differentiation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein an induction of cell differentiation indicates that the histone deacetylase isoform is required for the induction of cell proliferation.

43. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

44. A method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a histone deacetylase small molecule inhibitor that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor.

45. A method for modulating cell proliferation or differentiation of a cell comprising inhibiting a specific HDAC isoform that is involved in cell proliferation or differentiation by contacting the cell with an agent of claim 1.

46. The method according to claim 45, wherein the cell proliferation is neoplasia.

47. The method according to claim 46, wherein the histone deacetylase isoform is selected from the group consisting of HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8.

48. The method according to claim 47, wherein the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

MAQTQGTRRKVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLLN

YGLYRKMEIYRPHKANAEEMTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPV

FDGLFEFCQLSTGGSVASAVKLNKQQTDIAVNWAGGLHHAKKSEASGFCYVNDIVLAI

LELLKYHQRVLYIDIDIHHGDGVEEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGK

GKYYAVYPLRDGIDDESYEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNL

TIKGHAKCVEFVKSFNLPMLMLGGGGYTIRNVARCWTYETAVALDTEIPNELPYNDYF

EYFGPDFKLHISPSNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPEE

SGDEDEDDPDKRISICSSDKRIACEEEFSDSEEEGEGGRKNSSNFKKAKRVKTEDEKE

KDPEEKKEVTEEEKTKEEKPEAKGVKEEVKLA (SEQ ID NO:1)

FIG. 1A

```
   1 atgtctgggg tctctgcccg ctggtgctgc tgtctcccac tcggtcatcc tgagaacaca
  61 gcctgagcgr ctctgtcact cggggtagac cacgcgggga ggcgagcaag atggcgcaga
 121 cgcagggcac ccggaggaaa gtctgttact actacgacgg ggatgttgga aattactatt
 181 atggacaagg ccacccaatg aagcctcacc gaatccgcat gactcataat ttgctgctca
 241 actatggtct ctaccgaaaa atggaaatct atcgccctca caaagccaat gctgaggaga
 301 tgaccaagta ccacagcgat gactacatta aattcttgcg ctccatccgt ccagataaca
 361 tgtcggagta cagcaagcag atgcagagat tcaacgttgg tgaggactgt ccagtattcg
 421 atggcctgtt tgagttctgt cagttgtcta ctggtggttc tgtggcaagt gctgtgaaac
 481 ttaataagca gcagacggac atcgccgtga attgggctgg gggcctgcac catgcaaaga
 541 agtccgaggc atctggcttc tgttacgtca atgatatcgt cttggccatc ctggaactgc
 601 taaagtatca ccagagggtg ctgtacattg acattgatat tcaccatggt gacggcgtgg
 661 aagaggcctt ctacaccacg gaccgggtca tgactgtgtc ctttcataag tatggagagt
 721 acttcccagg aactggggac ctacgggata ccggggctgg caaagacaag tattatgctg
 781 ttaactaccc gctccgagac gggattgatg acgagtccta tgaggccatt ttcaagccgg
 841 tcatgtccaa agtaatggag atgttccagc ctagtgcggt ggtcttacag tgtggctcag
 901 actccctatc tggggatcgg ttaggttgct tcaatctatc tatcaaagga cacgccaagt
 961 gtgtggaatt tgtcaagagc tttaacctgc ctatgctgat gctgggaggc ggtggttaca
1021 ccattcgtaa cgttgcccgg tgctggacat atgagacagc tgtggccctg gatacggaga
1081 tccctaatga gcttccatac aatgactact ttgaatactt tggaccagat ttcaagctcc
1141 acatcagtcc ttccaatatg actaaccaga acacgaatga gtacctggag aagatcaaac
1201 agcgactgtt tgagaacctt agaatgctgc cgcacgcacc tggggtccaa acgcaggcga
1261 ttcctgagga cgccatccct gaggagagtg gcgatgagga cgaagacgac cctgacaagc
1321 gcatctcgat ctgctcctct gacaaacgaa ttgcctgtga ggaagagttc tccgattctg
1381 aagaggaggg agaggggggc cgcaagaact cttccaactt caaaaaagcc aagagagtca
1441 aaacagagga tgaaaaagag aaagacccag aggagaagaa aggaatcacc gaagaggaga
1501 aaaccaagga ggagaagcca gaagccaaag gggtcaagga ggaggccaag ttggcctgaa
1561 tggacctctc cagctctggc ttcctgctga gtccctcacg tttctttccc c (SEQ ID NO:2)
```

FIG. 1B

MAYSQGGGKKKCKVCYYYDGDIGNYYYGQGHPMKPHRIRMTHNLLL

NYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMHIPFNVGEDCP

AFDGLFEFCQLSTGGSVAGAVKLNRQQTDMAVNWAGGLHHAKKYEASGFCYVNDIVLA

ILELLKYHQRVLYIDIDIHHRGDGVEEAFYTTDRVMTVSFYGEYFPGTGDLRDIGAG

KGKYYAVNFPMCDGIDDESYGQIFKPIISKVMEMYQPSAVVLQCGADSLSGDRLGCFN

LTVKGHAKCVEVVKTFNLPLLMLGGGGYTILRNVARCWTYETAVALDCEIPNELPYNDY

FEYFGPDFKLHISPSNMTNQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHE

DSGDEDGEDPDKRISIRASDKRIACDEEFSDSEDEGEGGRNVADHKKGAKARIEED

KKETEDKKTDVKEEDKSKDNSGEKTDTKGTKSEQLSNP (SEQ ID NO:3)

FIG. 2A

```
   1 cgccgagctt tcggcacctc tgccgggtgg taccgagcct tcccggcgcc ccctcctctc
  61 ctcccaccgg cctgcccttc cccgcgggac tatcgccccc acgtttccct cagccctttt
 121 ctctcccggc cgagccgcgg cggcagcagc agcagcagca gcagcaggag gaggagcccg
 181 gtggcggcgg tggccgggga gcccatggcg tacagtcaag gaggcggcaa aaaaaaagtc
 241 tgctactact acgacggtga tattggaaat tattattatg gacagggtca tcccatgaag
 301 cctcatagaa tccgcatgac ccataacttg ctgttaaatt atggcttaca cagaaaaatg
 361 gaaatatata ggccccataa agccactgcc gaagaaatga caaaatatca cagtgatgag
 421 tatatcaaat ttctacggtc aataagacca gataacatgt ctgagtatag taagcagatg
 481 catatattta atgttggaga agattgtcca gcgtttgatg gactctttga gttttgtcag
 541 ctctcaactg gcggttcagt tgctggagct gtgaagttaa accgacaaca gactgatatg
 601 gctgttaatt gggctggagg attacatcat gctaagaaat acgaagcatc aggatcctgt
 661 tacgttaatg atattgtgct tgccatcctt gaattactaa agtatcatca gagagtctta
 721 tatatcgata tagatattca tcatggtgat ggtgtcgaag aagcttttta tacaacagat
 781 cgtgtaatga cggtatcatt ccataaatat ggggaatact ttcctggcac aggagacttg
 841 agggatattg gtgctggaaa aggcaaatac tatgctgtca attttccaat gtgtgatggt
 901 atagacgatg agtcatatgg gcagatattt aagcctatta tctcaaaggt gatggagatg
 961 tatcaaccta gtgctgtggt attacagtgt ggtgcagact cattatctgg tgatagactg
1021 ggttgtttca atctaacagt caaaggtcat gctaaatgtg tagaagttgt aaaaactttt
1081 aacttaccat tactgatgct tggaggaggt ggctacacaa tccgtaatgt tgctcgatgt
1141 tggacatatg agactgcagt tgcccttgat tgtgagattc ccaacgagtt gccatataat
1201 gattactttg agtattttgg accagacttc aaactgcata ttagtccttc aaacatgaca
1261 aaccagaaca ctccagaata tacggaaaag ataaaacagc gtttgtttga aaatttgcgc
1321 atgttacctc atgcacctgg tgtccagatg caagctattc cagaagatgc tgttcatgaa
1381 gacagtggag atgaagatgg agaagatcca gacaagagaa tttctattcg agcatcagac
1141 aagcggatag cttgtgatga agaattctca gattctgagg atgaaggaga aggaggtcga
1501 agaaatgtgg ctgatcataa gaaaggagca aagaaagcta gaattgaaga agataagaaa
1561 gaaacagagg acaaaaaaac agacgttaag gaagaagata aatccaagga caacagtggt
1621 gaaaaaacag ataccaaagg aaccaaatca gaacagctca gcaacccctg aatctgacag
1681 tctcaccaat ttcagaaaat cattaaaaag aaaatattga aaggaaaatg ttttcttttt
1741 gaagacttct ggcttcattt tatactactt tggcatggac tgtatttatt ttcaaatggg
1801 acttttttcgt ttttgttttt ctgggcaagt tttattgtga gattttctaa ttatgaagca
1861 aaatttcttt tctccaccat gctttatgtg atagtattta aaattgatgt gagttattat
1921 gtcaaaaaaa ctgatctatt aaagaagtaa ttggcctttc tgagctgaaa aaaaaaaaaa
1981 aaaag (SEQ ID NO:4)
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FIG. 2B

MAKTVAYFYDPDVGNFHYGAGHPMKPHRLALTHSLVLHYGLYKK

MIVFKPYQASQHDMCRFHSEDYIDFLQRVSPTNMQGFTKSLNAPNVGDDCPVFPGLFE

FCSRYTGASLQGATQLNNKICDIANWAGGLHHAKKFEASGFCYVNDIVIGILELLLKY

HPRVLYIDIDIHHGDGVQEAFYLTDRVMTVSFHKYGNYFFPGTGDMYEVGAESGRYYC

LNVPLRDGIDDQSYKHLFQPVINQVVDFYQPTCIVLQCGADSLGCDRLGCFNLSIRGH

CECVEYVKSFNIPPLLVLGGGGYTVRNVARCWTYETSLLVEEAISEELPYSEYFEYFAP

DFTLHPDVSTRIENQSRQYLDQIRQTIFENLKMLNHAPSVQIHDVPADLLTYDRTDE

ADAEERGPEENYSRPEAPNEFYDGDHDNDKESDVEI (SEQ ID NO:5)

FIG. 3A

```
   1 ggaattcgcg gccgcggcgg gcgcgggagg tgcggggcct gctcccgccg gcaccatggc
  61 caagaccgtg gcctatttct acgaccccga cgtgggcaac ttccactacg gagctggaca
 121 ccctatgaag ccccatcgcc tggcattgac ccatagcctg gtcctgcatt acggtctcta
 181 taagaagatg atcgtcctca agccatacca ggcctcccaa catgacatgt gccgcttcca
 241 ctccgaggac tacattgact tcctgcagag agtcagcccc accaatatgc aaggcttcac
 301 caagagtctt aatgccttca acgtaggcga tgactgccca gtgtttcccg ggctctttga
 361 gttctgctcg cgttacacag gcgcatctct gcaaggagca acccagctga acaacaagat
 421 ctgtgatatt gccattaact gggctggtgg tctgcaccat gccaagaagt ttgaggcctc
 481 tggcttctgc tatgtcaacg acattgtgat tggcatcctg gagctgctca agtaccaccc
 541 tcgggtgctc tacattgaca ttgacatcca ccatggtgac ggggttcaag aagctttcta
 601 cctcactgac cgggtcatga cggtgtcctt ccacaaatac ggaaattact tcttccctgg
 661 cacaggtgac atgtatgaag tcggggcaga gagtggccgc tactactgtc tgaacgtgcc
 721 cctgcgggat ggcattgatg accagagtta caagcacctt ttccagccgg ttatcaacca
 781 ggtagtggac ttctaccaac ccacgtgcat tgtgctccag tgtggagctg actctctggg
 841 ctgtgatcga ttgggctgct ttaacctcag catccgaggg catggggaat gcgttgaata
 901 tgtcaagagc ttcaatatcc ctctactcgt gctgggtggt ggtggttata ctgtccgaaa
 961 tgttgcccgc tgctggacat atgagacatc gctgctggca gaagaggcca ttagtgagga
1021 gcttccctat agtgaatact tcgagtactt tgccccagac ttcacacttc atccagatgt
1081 cagcacccgc atcgagaatc agaactcacg ccagtatctg gaccagatcc gccagacaat
1141 ctttgaaaac ctgaagatgc tgaaccatgc acctagtgtc cagattcatg acgtgcctgc
1201 agacctcctg acctacgaca ggaccgatga ggccgatgca gaggagaggg gtcctgagga
1261 gaactatagc aggccagagg catccaatga gttctatgat ggagaccatg acaatgacaa
1321 ggaaagcgat gtggagattt aagagtggct tgggatgctg tgtcccaagg aatttctttt
1381 cacctcttgg aagggctgga gggaaaagga gtggctccta gagtcctggg ggtcacccca
1441 ggggcttttg ctgactctgg gaaagagtct ggagaccaca tttggttctc gaaccatcta
1501 cctgcttttc ctctctctcc caaggactga caatggtacc tattagggat gagatacaga
1561 caaggatagc tatctgggac attattggca gtgggccctg gaggcagtcc ctagcccccc
1621 ttgcccctta tttcttccct gcttccctcg aacccagaga tttttgaggg atgaacgggt
1681 agacaaggac tgagattgcc tctgacttcc tcctcccctg ggttctgacc ttcttcctcc
1741 ccttgcttcc agggaagatg aagagagaga gatttggaag ggctctggc tccctaacac
1801 ctgaatccca gatgatggga agtatgtttt caagtgtggg gaggatatga aaatgttctg
1861 ctctcacttt tggctttatg tccattttac cactgttttt atccaataaa ctaagtcggt
1921 atttttgta cctttgatgg tttagcggcc gcgc (SEQ ID NO:6)
```

FIG. 3B

MLAMKHQQELLEHQRKLERHRQEQELEKQHREQKLQQLKNKEKG

KESAVASTEVKMKLQEFVLNKKKALAHPNLNHCISSCPRYWYGKTQHSSLDQSSPPQS

GVSTSYNHPVLGMYDAKDDFPLRKTASEPNLKLRSRLKQKVAERRSSPLLRRKDGPVV

TALKKRPLDVTDSACSSAPGSGPSSPNNSSGSVSAENGIAPAVPSIPAETSLAHRLVA

REGSAAPLPLYTSPSLPNITLGLPATGPSAGTAGQQDTERLTLPALQQRLSLFPGTHL

TPYLSTSPLERDGGAAHSPLLQHMVLLEQPPAQAPLVTGLGALPLHAQSLVGADRVSP

SIHKLRQHRPLGRTQSAPLPQNAQALQHLVIQQQHQQFLEKHKQQFQQQQLQMNKIIP

KPSEPARQPESHPEETEEELREHQALLDEPYLDRLPGQKEAHAQAGVQVKQEPIESDE

EEAEPPREVEPGQRQPSEQELLFRQQALLLEQQRIHQLRNYQASMEAAGIPVSFGGHR

PLSRAQSSPASATFPVSVQEPPTKPRFTTGLVYDTLMLKHQCTCGSSSSHPEHAGRIQ

SIWSRLQETGLRGKCECIRGRKATLEELQTVHSEAHTLLYGTNPLNRQKLDSKKLLGS

LASVFVRLPCGGVGVDSDTIWNEVHSAGAARLAVGCVVELVFKVATGELKNGFAVVRP

PGHHAEESTPMGFCYFNSVAVAAKLLQQRLSVSKILIVDWDVHHGNGTQQAFYSDPSV

LYMSLHRYDDGNFFPGSGAPDEVGTGPGVGFNVNMAFTGGLDPPMGDAEYLAAFRTVV

MPIASEFAPDVVLASSGFDAVEGHPTPLGGYNLSARCFGYLTKQLMGLAGGRIVLALE

GGHDLTAICDASEACVSALLGNELDPLPEKVLQQRPNANAVRSMEKVMEIHSKYWRCL

QRTTSTAGRSLIEAQTCENEEAETVTAMASLSVGVKPAEKRPDEEPMEEEPPL (SEQ ID NO:7)

FIG. 4A

```
  1 ggaggttgtg gggccgccgc cgcggagcac cgtccccgcc gccgcccgag cccgagcccg
 61 agcccgcgca cccgcccgcg ccgccgccgc cgccgcccga acagcctccc agcctgggcc
121 cccggcggcg ccgtggccgc gtcccggctg tcgccgcccg agcccgagcc cgcgcgccgg
181 cgggtggcgg cgcaggctga ggagatgcgg cgcggagcgc cggagcaggg ctagagccgg
241 ccgccgccgc ccgccgcggt aagcgcagcc ccggcccggc gcccgcgggc cattgtccgc
301 cgcccgcccc gcgccccgcg cagcctgcag gccttggagc ccgcggcagg tggacgccgc
361 cggtccacac ccgccccgcg cgcggccgtg ggaggcgggg gccagcgctg gccgcgcgcc
421 gtgggacccg ccggtcccca gggccgcccg gccccttctg gacctttcca cccgcgccgc
491 gaggcggctt cgcccgccgg ggcgggggcg cgggggtggg cacggcaggc agcggcgccg
541 tctcccggtg cggggcccgc gcccccccgag caggttcatc tgcagaagcc agcggacgcc
601 tctgttcaac ttgtgggtta cctggctcat gagaccttgc cggcgaggct cggcgcttga
661 acgtctgtga cccagccctc accgtcccgg tacttgtatg tgttggcggg agtttggagc
721 tcgttggagc tatcgtttcc gtggaaattt tgagccattt cgaatcactt aaaggagtgg
781 acattgctag caatgagctc ccaaagccat ccagatggac tttctggccg agaccagcca
841 gtggagctgc tgaatcccgc ccgcgtgaac cacatgccca gcacggtgga tgtggccacg
901 gcgctgcctc tgcaagtggc ccccccggca gegcccatgg acccgcgcct ggaccaccag
961 ttctcactgc ctgtggcaga gccggccctg cgggagcagc agctgcagca ggagctcctg
```

FIG. 4B-1

```
1021 gcgctcaagc agaagcagca gatccagagg cagatcctca tcgccgagtt ccagaggcag
1081 cacgagcagc tctcccggca gcacgaggcg cagctccacg agcacatcaa gcaataacag
1141 gagatgctgg ccatgaagca ccagcaggag ctgctggaac accagcggaa gctggagagg
1201 caccgccagg agcaggagct ggagaagcag caccgggagc agaagctgca gcagctcaag
1261 aacaaggaga agggcaaaga gagtgccgtg gccagcacag aagtgaagat gaagttacaa
1321 gaatttgtcc tcaataaaaa gaaggcgctg gcccaccgga atctgaacca ctgcacttcc
1381 agagaccctc gctactggta cgggaaaacg cagcacagtt cccttgacca gagttctcca
1441 ccccagagcg gagtgtcgac ctcctataac cacccggtcc tgggaatgta cgacgccaaa
1501 gatgacttcc ctcttaggaa aacagcttct gaaccgaatc tgaaatcacg gtccaggcta
1561 aagcagaaag tggccgaaag acggagcagc cccctgttac gcaggaaaga cgggccagtg
1621 gtcactgctc taaaaaagcg tccgttggat gtcacagact ccgcgtgcag cagcgcccca
1681 ggctccggac ccagctcacc caacaacagc tccgggagcg tcgcgtggag gaacggtatc
1741 gcgcccgccg tccccagcat cccggcggag acgagtttgg cgcacagact tgtggcacga
1801 gaaggctcgg ccgctccact tcccctctac acatcgccat ccttgcccaa catcacgctg
1861 ggcctgcctg ccaccggccc ctctgcgggc acggcgggcc agcaggacac cgagagactc
1921 acccttcccg ccctccagca gaggctctcc cttttccccg gcacccacct cactccctac
1981 ctgagcacct cgcccttgga gcgggacgga ggggcagcgc acagccctct tctgcagcac
2041 atggtcttac tggagcagcc accggcacaa gcacccctcg tcacaggcct gggagtactg
2101 cccctccacg cacagtcctt ggttggtgca gaccgggtgt ccccctccat ccacaagctg
2161 cggcagcacc gcccactggg gcggacccag tcggccccgc tgccccagaa cgcccaggct
2221 ctgcagcacc tggtcatcca gcagcagcat cagcagtttc tggagaaaca caagcagcag
2281 ttccagcagc agcaactgca gatgaacaag atcatcccca agccaagcga gccagcccgg
2341 cagccggaga gccacccgga ggagacggag gaggagctcc gtgagcacca ggctctgctg
2401 gacgagccct acctggaccg gctgccgggg cagaaggagg cgcacgcaca ggccggcgtg
2461 caggtgaagc aggagcccat tgagagcgat gaggaagagg cagagccccc acgggaggtg
2521 gagccgggcc agcgccagcc cagtgagcag gagctgctct tcagacagca agccctcctg
2581 ctggagcagc agcggatcca ccagctgagg aactaccagg cgtccatgga ggccgccggc
2641 atccccgtgt ccttcggcgg ccacaggcct ctgtcccggg cgcagtcctc acccgcgtct
2701 gccaccttcc ccgtgtccgt gcaggagccc cccaccaagc cgaggttcac gacaggcctc
2761 gtgtatgaca cgctgatgct gaagcaccag tgcacctgcg ggagtagcag cagccacccc
2821 gagcacgccg ggaggatcca gagcatctgg tcccgcctgc agaagacggg cctccggggc
```

FIG. 4B-2

SUBSTITUTE SHEET (RULE 26)

2881 aaatgcgagt gcatccgcgg acgcaaggcc accctggaag agctacagac ggtgcactcg
2941 gaagcccaca ccctcctgta tggcacgaac cccctcaacc ggcagaaact ggacagtaag
3001 aaacttctag gctcgctcgc ctccgtgttc gtccggctcc cttgcggtgg tgttggggtg
3061 gacagtgaca ccatatggaa cgaggtgcac tcggcgggggcagcccgcct ggctgtgggc
3121 tgcgtggtag agctggtctt caaggtggcc acaggggagc tgaaaaatgg ctttgctgtg
3181 gtccgccccc ctggacacca tgcggaggag agcacgccca tgggcttttg ctacttcaac
3241 tccgcggccg tggcagccaa gcttctgcag cagaggttga gcgtgagcaa gatcctcatc
3301 gtggactggg acgtgcacca tggaaacggg acccagcagg ctttctacag cgaccctagc
3361 gtcctgtaca tgtccctcca ccgctacgac gatgggaact tcttcccagg cagcggggct
3421 cctgatgagg tgggcacagg gcccggcgtg ggtttcaacg tcaacacggc tttcaccggc
3481 ggcctggacc cccccatggg agacgctgag tacttggcgg ccttcagaac ggtggtaatg
3541 ccgatcgcca gcgagtttgc cccggatgtg gtgctggtgt catcaggctt cgatgccgtg
3601 gagggccacc ccacccctct tggggggctac aacctctccg ccagatgctt cgggtacctg
3661 acgaagcagc tgatgggcct ggctggcggc cggattgtcc tggccctcga gggaggccac
3721 gacctgaccg ccatttgcga cgcctcggaa gcatgtgttt ctgccttgct gggaaacgag
3781 cttgatcctc tcccagaaaa ggttttacag caaagaccca atgcaaacgc tgtccgttcc
3841 atggagaaag tcatggagat ccacagcaag tactggcgct gcctgcagcg cacaacctcc
3901 acagcggggc gttctctgat cgaggctcag acttgcgaga acgaagaagc cgagacggtc
3961 accgccatgg cctcgctgtc cgtggacgtg aagcccgccg aaaagagacc agatgaggag
4021 cccatggaag aggagccgcc cctgtagcac tccctcgaag ctgctgttct cttgtctgtc
4081 tgtctctgtc ttgaagctca gccaagaaac tttcccgtgt cacgcctgcg tcccaccgtg
4141 gggctctctt ggagcaccca gggacaccca gcgtgcaaca gccacgggaa gcctttctgc
4201 cgcccaggcc cacaggtctc gagacgcaca tgcacgcctg ggcgtggcag cctcacaggg
4261 aacacgggac agacgccggc gacgcgcaga cacacggaca cgcggaagcc aagcacactc
4321 tggcgggtcc cgcaagggac gccgtggaag aaaggagcct gtggcaacag gcggccgagc
4381 tgccgaattc agttgacacg aggcacagaa aacaaatatc aaagatctaa taatacaaaa
4441 caaacttgat taaaactggt gcttaaagtt tattacccac aactccacag tctctgtgta
4501 aaccactcga ctcatcttgt agcttatttt tttttaaag aggacgtttt ctacggctgt
4561 ggcccgcctc tgtgaaccat agcggtgtgc ggcgggggt ctgcacccgg gtgggggaca
4621 gagggacctt taaagaaaac aaaactggac agaaacagga atgtgagctg ggggagctgg
4681 cttgagtttc tcaaaagcca tcggaagatg cgagtttgtg cctttttttt tattgctctg

FIG. 4B-3

SUBSTITUTE SHEET (RULE 26)

FIG. 4B-4

```
4741 gtggattttt gtggctgggt tttctgaagt ctgaggaaca atgccttaag aaaaaacaaa
4801 cagcaggaat cggtgggaca gtttcctgtg gccagccgag cctggcagtg ctggcaccgc
4861 gagctggcct gacgcctcaa gcacgggcac cagccgtcat ctccggggcc aggggctgca
4921 gcccggcggt ccctgttttg ctttattgct gtttaagaaa aatggaggta gttccaaaaa
4981 agtggcaaat cccgttggag gttttgaagt ccaacaaatt ttaaacgaat ccaaagtgtt
5041 ctcacacgtc acatacgatt gagcatctcc atctggtcgt gaagcatgtg gtaggcacac
5101 ttgcagtgtt acgatcggaa tgctttttat taaaagcaag tagcatgaag tattgcttaa
5161 attttaggta taaataaata tatatatgta taatatatat tccaatgtat tccaagctaa
5221 gaaacttact tgattcttat gaaatcttga taaaatattt ataatgcatt tatagaaaaa
5281 gtatatatat atatataaaa tgaatgcaga ttgcgaaggt ccctgcaaat ggatggcttg
5341 tgaatttgct ctcaaggtgc ttatggaaag ggatcctgat tgattgaaat tcatgttttc
5401 tcaagctcca gattggctag atttcagatc gccaacacat tcgccactgg gcaactaccc
5461 tacaagtttg tactttcatt ttaattattt tctaacagaa ccgctcccgt ctccaagcct
5521 tcatgcacat atgtacctaa tgagttttta tagcaaagaa tataaatttg ctgttgattt
5561 ttgtatgaat tttttcacaa aaagatcctg aataagcatt gttttatgaa ttttacattt
5641 ttcctcacca tttagcaatt ttccgaatgg taataatgtc taaatctttt tcctttctga
5701 attcttgctt gtacattttt ttttaccttt caaaggtttt taattatttt tgtttttatt
5761 tttgtacgat gagttttctg cagcgtacag aattgttgct gtcagattct attttcagaa
5821 agtgagagga gggaccgtag gtcttttcgg agtgacacca acgattgtgt ctttcctggt
5881 ctgtcctagg agctgtataa agaagcccag gggctctttt taactttcaa cactagtagt
5941 attacgaggg gtggtgtgtt tttcccctcc gtggcaaggg cagggagggt tgcttaggat
6001 gcccggccac cctgggaggc ttgccagatg ccgggggcag tcagcattaa tgaaactcat
6061 gtttaaactt ctctgaccac atcgtcagga tagaattcta acttgagttt tccaaacacc
6121 ttttgagcat gtcagcaatg catggggcac acgtggggct ctttacccac ttgggttttt
6181 ccactgcagc cacgtggcca gccctggatt ttggagcctg tggctgcaag gaacccaggg
6241 acccttgttg cctggtgaac ctgcagggag ggtatgattg cctgaccagg acagccagtc
6301 tttactcttt ttctcttcaa cagtaactga cagtcacgtt ttactggtaa cttattttcc
6361 agcacatgaa gccaccagtt tcattccaaa gtgtatattg ggttcagact tgggggcaga
6421 agttcagaca caccgtgctc aggagggacc cagagccgag tttcggagtt tggtaaagtt
6481 tacagggtag cttctgaaat taactcaaac ttttgaccaa atgagtgcag attcttggat
6541 tcacttggtc actgggctgc tgatggtcag ctctgagaca gtggtttgag agcaggcaga
```


SUBSTITUTE SHEET (RULE 26)

FIG. 4B-5

```
6601 acggtcttgg gacttgtttg actttcccct ccctggtggc cactctttgc tctgaagccc
6661 agattggcaa gaggagctgg tccattcccc attcatggca cagaacagtg gcagggccca
6721 gctagcaggc tcttctggcc tccttggcct cattctctgc atagccctct ggggatcctg
6781 ccacctgccc tcttaccccg ccgtggctta tggggaggaa tgcatcatct cacttttttt
6841 ttttaagcag atgatgggat aacatggact gctcagtggc caggttatca gtgggggggac
6901 ttaattctaa tctcattcaa atggagacga cctctgcaaa ggcctggcag ggggaggcaa
6961 gtttcatctg tcagctcact ccagcttcac aaatgtgctg agagcattac tgtgtagcct
7021 tttctttgaa gacacactcg gctcttctcc acagcaagcg tccagggcag atggcagagg
7081 atctgcctcg gcgtctgcag gcgggaccac gtcagggagg gttccttcat gtgttctccc
7141 tgtgggtcct tggaccttta gcctttttct tcctttgcaa aggccttggg ggcactggct
7201 gggagtcagc aagcgagcac tttatatccc tttgagggaa accctgatga cgccactggg
7261 cctcttggcg tctgacctgc cctcgccgct tcccgccgtg ccgcagcgtg cccacgtgcc
7321 cacgccccac cagcaggcgg ctgccccgga ggccgtggcc cgctgggact ggccgcccct
7381 ccccagcgtc ccagggctct ggttctggag ggccactttg tcaaggtgtt tcagtttttc
7441 tttacttctt ttgaaaatct gtttgcaagg ggaaggacca tttcgtaatg gtctgacaca
7501 aaagcaagtt tgatttttgc agcactagca atggactttg ttgcttttct ttttgatcag
7561 aacattcctt ctttactggt cacagccacg tgctcattcc attcttcttt ttgtagactt
7621 tgggcccacg tgttttatgg gcattgatac atatataaat atatagatat aaatatatat
7681 gaatacattt ttttaagttt cctacacctg gaggttgcat ggactgtacg accggcatga
7741 ctttatattg tatacagatt ttgcacgcca aactcggcag ctttggggaa gaagaaaaat
7801 gcctttctgt tcccctctca tgacatttgc agatacaaaa gatggaaatt tttctgtaaa
7861 acaaaacctt gaaggagagg agggcgggga agtttgcgtc ttattgaact tattcttaag
7921 aaattgtact ttttattgta agaaaaataa aaaggactac ttaaacattt gtcatattaa
7981 gaaaaaaagt ttatctagca cttgtgacat accaataata gagtttattg tatttatgtg
8041 gaaacagtgt tttagggaaa ctactcagaa ttcacagtga actgcctgtc tctctcgagt
8101 tgatttggag gaattttgtt ttgttttgtt ttgtttgttt ccttttatct ccttccacgg
8161 gccaggcgag cgccgcccgc cctcactggc cttgtgacgg tttattctga ttgagaactg
8221 ggcggactcg aaagagtccc cttttccgca cagctgtgtt gactttttaa ttacttttag
8281 gtgatgtatg gctaagattt cactttaagc agtcgtgaac tgtgcgagca ctgtggttta
8341 caattatact ttgcatcgaa aggaaaccat ttcttcattg taacgaagct gagcgtgttc
8401 ttagctcggc ctcactttgt ctctggcatt gattaaaagt ctgctattga aagaaaaag(SEQ ID NO:8)
```


SUBSTITUTE SHEET (RULE 26)

LRQGGTLTGKFMSTSSIPGCLLGVALEGDGSPHGHASLLQHVLL

LEQARQQSTLIAVPLHGQSPLVTGERVATSMRTVGKLPRHRPLSRTQSSPLPQSPQAL

QQLVMQQQHQQFLEKQKQQQLQLGKILTKTGELPRQPTTHPEETEEELTEQQEVLLGE

GALTMPREGSTESESTQEDLEEEDEEEDGEEEDCIQVKDEEGESGAEEGPDLEEPGA

GYKKLFSDAQPLQPLQVYQAPLSLATVPHQALGRTQSSPAAPGGMKSPPDQPVKHLFT

TGVVYDTFMLKHQCMCGNTHVHPEHAGRIQSIWSRLQETGLLSKCERIRGRKATLDEI

QTVHSEYIHTLLYGTSPLNRQKLDSKKLLGPISQKMYAVLPCGGIGVDSDTVWNEMHSS

SAVRMAVGCLLELAFKVAAGELKNGFAIIRPPGHHAEESTAMGFCFFNSVAITAKLLQ

QKLNVGKVLIVDWDIHHGNGTQQAFYNDPSVLYISLHRYDNGNFFPGSGAPEEVGGGP

GVGYNVNVAWTGGVDPPIGDVEYLTAFRTVVMPIAHEFSPDVVTLVSAGFDAVEGHLSP

LGGYSVTARCFGHLTRQLMTLAGGRVVLALEGGHDLTAICDASEACVSALLSVELQPL

DELVLQQKPNINAVATLEKVIETQSKHWSCVQKFAAGLGRSLREAQAGETEEAETVSA

MALLSVGAEQAQAAAAREHSPRPAEEPMEQEPAL (SEQ ID NO:9)

FIG. 5A

```
  1 ccctgcggca gggtggcacg ctgaccggca agttcatgag cacatcctct attcctggct
 61 gcctgctggg cgtggcactg gagggcgacg ggagccccca cgggcatgcc tccctgctgc
121 agcatgtgct gttgctggag caggcccggc agcagagcac cctcattgct gtgccactcc
181 acgggcagtc cccactagtg acgggtgaac gtgtggccac cagcatgcgg acggtaggca
241 agctcccgcg gcatcggccc ctgagccgca ctcagtcctc accgctgccg cagagtcccc
301 aggccctgca gcagctggtc atgcaacaac agcaccagca gttcctggag aagcagaagc
361 agcagcagct acagctgggc aagatcctca ccaagacagg ggagctgccc aggcagccca
421 ccacccaccc tgaggagaca gaggaggagc tgacggagca gcaggaggtc ttgctggggg
481 agggagccct gaccatgccc cgggagggct ccacagagag tgagagcaca caggaagacc
541 tggaggagga ggacgaggaa gaggatgggg aggaggagga ggattgcatc caggttaagg
601 acgaggaggg cgagagtggt gctgaggagg ggcccgactt ggaggagcct ggtgctggat
661 acaaaaaact gttctcagat gcccagccgc tgcagccttt gcaggtgtac caggcgcccc
721 tcagcctggc cactgtgccc caccaggccc tgggccgtac ccagtcctcc cctgctgccc
781 ctgggggcat gaagagcccc ccagaccagc ccgtcaagca cctcttcacc acaggtgtgg
841 tctacgacac gttcatgcta aagcaccagt gcatgtgcgg gaacacacac gtgcaccctg
```

FIG. 5B-1

```
 901 agcatgctgg ccggatccag agcatctggt cccggctgca ggagacaggc ctgcttagca
 961 agtgcgagcg gatccgaggt cgcaaagcca cgctagatga gatccagaca gtgcactctg
1021 aataccacac cctgctctac gggaccagtc ccctcaaccg gcagaagcta gacagcaaga
1081 agttgctcgg ccccatcagc cagaagatgt atgctgtgct gccttgtggg ggcatcgggg
1141 tggacagtga caccgtgtgg aatgagatgc actcctccag tgctgtgcgt atggcagtgg
1201 gctgcctgct ggagctggcc ttcaaggtgg ctgcaggaga gctcaagaat ggatttgcca
1261 tcatccggcc cccaggacac cacgccgagg aatccacagc cacgggattc tgcttcttca
1321 actctgtagc catcaccgca aaactcctac agcagaagtt gaacgtgggc aaggtcctca
1381 tcgtggactg ggacattcac catggcaatg gcacccagca ggcgttctat aatgacccct
1441 ctgtgctcta catctctctg catcgctatg acaacgggaa cttctttcca ggctctgggg
1501 ctcctgaaga ggttggtgga ggaccaggcg tggggtacaa tgtgaacgtg gcatggacag
1561 gaggtgtgga ccccccatt ggagacgtgg agtaccttac agccttcagg acagtggtga
1621 tgcccattgc ccacgagttc tcacctgatg tggtcctagt ctccgccggg tttgatgctg
1681 ttgaaggaca tctgtctcct ctgggtggct actctgtcac cgccagatgt tttggccact
1741 tgaccaggca gctgatgacc ctggcagggg gccgggtggt gctggccctg gagggaggcc
1801 atgacttgac cgccatctgt gatgcctctg aggcttgtgt ctcggctctg ctcagtgtag
1861 agctgcagcc cttggatgag gcagtcttgc agcaaaagcc caacatcaac gcagtggcca
1921 cgctagagaa agtcatcgag atccagagca aacactggag ctgtgtgcag aagttcgccg
1981 ctggtctggg ccggtccctg cgagaggccc aagcaggtga ggccgaggag gccgagactg
2041 tgagcgccat ggccttgctg tcggtggggg ccgagcaggc ccaggctgcg gcagcccggg
2101 aacacagccc caggccggca gaggagccca tggagcagga gcctgccctg tgacgccccg
2161 gcccccatcc ctctcggctt caccattgtg atttgtttta ttttttctat taaaaacaaa
2221 aagtcacaca ttc (SEQ ID NO:10)
```

FIG. 5B-2

```
   1 mtstgqdstt trqrrsrqnp qsppqdssvt skrnikkgav prsipnlaev kkkgkmkklg
  61 qameedlivg lqgmdlnlea ealagtglvl deqlnefhcl wddsfpegpe rlhaikeqli
 121 qeglldrcvs fqarfaekee lmlvhsleyi dlmettqymn egelrvladt ydsvylhpns
 181 yscaclasgs vlrlvdavlg aeirngmaii rppghhaqhs lmdgycmfnh vavaaryaqq
 241 khrirrvliv dwdvhhgqgt qftfdqdpsv lyfsihryeq grfwphlkas nwsttgfgqg
 301 qgytinvpwn qvgmrdadyi aaflhvllpv alefqpqlvl vaagfdalqg dpkgemaatp
 361 agfaqlthll mglaggklil sleggynira laegvsaslh tllgdpcpml espgapcrsa
 421 qasvscalea lepfwevlvr stetverdnm eednveesee egpweppvlp iltwpvlqsr
 481 tglvydqnmm nhcnlwdshh pevpqrilri morleelgia grcltitprp ateaelltch
 541 saeyvghlra tekmktrelh ressnfdsiy icpstfacaq Iatgaacrlv eavisgevin
 601 gaavvrppgh haeqdaacgf cffnsvavaa rhaqtisgha lrilivdwdv hhgngtqhmf
 661 eddpsvlyvs lhrydhgtff pmgdegassq igraagtgft vnvawngprm gdadylaawh
 721 rlvlpiayef npelvlvsag fdaargdplg gcqvspegya hlthllmgla sgriilileg
 781 gynltsises maactrsilg dppplltlpr pplsgalasi tetiqvhrry wrslrvmkve
 841 dregpssskl vtkkapqpak prlaermttr ekkvleagmg kvtsasfgee stpgqtnset
 901 avvalcqdqp seaatggatl aqtiseaaig gamlgqttse eavggatpdq ttseetvgga
 961 ildqttseda vggatiggtt seeavggatl aqtiseaame gatldqttse eapggteliq
1021 tplasstdhq tpptspvqgt tpqispstli gslrtlelgs esqgasesqa pgeenllgea
1081 aggqdmadsm lmqgsrgltd qaifyavtpl pwcphlvavc pipaagldvt qpcgdcgtiq
1141 enwvclscyq vycgryingh mlqhhgnsgh plvlsyidls awcyycqayv hhqalldvkn
1201 iahqnkfged mphph (SEQ ID:11)
```

FIG. 6A

```
  1 gggcagtccc ctgaggagcg gggctggttg aaacgctagg ggcgggatct ggcggagtgg
 61 aagaaccgcg gcaggggcca agcctcctca actatgacct caaccggcca ggattccacc
121 acaaccaggc agcgaagaag taggcagaac ccccagtcgc cccctcagga ctccagtgtc
181 acttcgaagc gaaatattaa aaagggagcc gttccccgct ctatccccaa tctagcggag
241 gtaaagaaga aaggcaaaat gaagaagctc ggccaagcaa tggaagaaga cctaatcgtg
301 ggactgcaag ggatggatct gaacctcgag gctgaagcac tggctggcac tggcttggtg
361 ttggatgagc agttaaatga attccattgc ctctgggatg acagcttccc ggaaggccct
421 gagcggctcc atgccatcaa ggagcaactg atccaggagg gcctcctaga tcgctgcgtg
481 tcctttcagg cccggtttgc tgaaaaggaa gagctgatgt tggttcacag cctagaatat
```

FIG. 6B-1

```
541  attgacctga tggaaacaac ccagtacatg aatgagggag aactccgtgt cctagcagac
601  acccacgact cagtttatct gcatccgaac tcatactcct gtgcctgcct ggcctcaggc
661  tctgtcctca ggctggtgga tgcggtcctg gggctgaga tccggaacgg catggccatc
721  attaggcctc ctggacatca cgcccagcac agtcttatgg atggctattg catgttcaac
781  cacgtggctg tggcagcccg ctatgctcaa cagaaacacc gcacccggag ggtccttatc
841  gtagattggg atgtgcacca cggtcaagga acacagttca ccttcgacca ggaccccagt
901  gtcctctatt tctccatcca ccgctacgag cagggtaggt tctggcccca cctgaaggcc
961  tctaactggt ccaccacagg tttcggccaa ggccaaggat ataccatcaa tgtgccttgg
1021 aaccaggtgg ggatgcggga tgctgactac attgctgctt tcctgcacgt cctgctgcca
1081 gtcgccctcg agctccagcc tcagctggtc ctggtggccg ctggatttga tgccctgcaa
1141 ggggacccca agggcgagat ggccgccact ccggcagggt tcgcccagct aacccacctg
1201 ctcatgggtc tggcaggagg caagctgatc ctgtctctgg agggtggcta caacctccgc
1261 gccctggctg aaggcgtcag tgcttcgctc cacacccttc tgggagaccc ttgccccatg
1321 ccggagtcac ctggtgcccc ctgccggagc gcccaggctt cagtttcctg tgctctggaa
1381 gcccttgagc ccttctggga ggttcttgtg agatcaactg agaccgtgga gagggacaac
1441 atggaggagg acaatgtaga ggagagcgag gaggaaggac cctgggagcc ccctgtgctc
1501 ccaatcctga ca1ggccagt gctacagtct cgcacagggc tggtctatga ccaaaatatg
1561 atgaatcact gcaacttgtg ggacagccac caccctgagg taccccagcg catcttgcgg
1621 atcatgtgcc gtctggagga gctgggcctt gccgggcgct gcctcaccct gacaccgcgc
1681 cctgccacag aggctgagct gctcacctgt cacagtgctg agtacgtggg tcatctccgg
1741 gccacagaga aaatgaaaac ccgggagctg caccgtgaga gttccaactt tgactccatc
1801 tatatctgcc ccagtacctt cgcctgtgca cagcttgcca ctggcgctgc ctgccgcctg
1861 gtggaggctg tgctctcagg agaggtcctg aatggtgctg ctgtggtgcg tcccccagga
1921 caccacgcag agcaggatgc agcttgcggt tttigctttt tcaactctgt ggctgtggct
1981 gctcgccatg cccagactat cagtgggcat gccctacgga tcctgattgt ggattgggat
2041 gtccaccacg gtaatggaac tcagcacatg tttgaggatg accccagtgt gctatatgtg
2101 tccctgcacc gctatgatca tggcaccttc ttccccatgg gggatgaggg tgccagcagc
2161 cagatcggcc gggccgcggg cacaggcttc accgtcaacg tggcatggaa cgggccccgc
2221 atgggtgatg ctgactacct agctgcctgg catcgcctgg tgcttcccat tgcctacgag
2281 tttaacccag aactggtgct ggtctcagct ggctttgatg ctgcacgggg ggatccgctg
```

FIG. 6B-2

```
2341 gggggctgcc aggtgtcacc tgagggttat gcccacctca cccacctgct gatgggcctt
2401 gccagtggcc gcattatcct tatcctagag ggtggctata acctgacatc catctcagag
2461 tccatggctg cctgcactcg ctccctcctt ggagacccac cacccCtgct gaccctgcca
2521 cggcccccac tatcaggggc cctggcctca atcactgaga ccatccaagt ccatcgcaga
2581 tactggcgca gcttacgggt catgaaggca gaagacagag aaggaccctc cagttctaag
2641 ttggtcacca agaaggcacc ccaaccagcc aaacctaggt tagctgagcg gatgaccaca
2701 cgagaaaaga aggttctgga agcaggcatg gggaaagtca cctcggcatc atttggggaa
2761 gagtccactc caggccagac taactcagag acagctgtgg tggccctcac tcaggaccag
2821 ccctcagagg cagccacagg gggagccact ctggcccaga ccatttctga ggcagccatt
2881 gggggagcca tgctgggcca gaccacctca gaggaggctg tcggggggagc cactccggac
2941 cagaccacct cagaggagac tgtgggagga gccattctgg accagaccac ctcagaggat
3001 gctgttgggg gagccacgct gggccagact acctcagagg aggctgtagg aggagctaca
3061 ctggcccaga ccatctcgga ggcagccatg gagggagcca cactggacca gactacgtca
3121 gaggaggctc caggggggcac cgagctgatc caaactcctc tagcctcgag cacagaccac
3181 cagacccccc caacctcacc tgtgcaggga actacacccc agatatctcc cagtacactg
3241 attgggagtc tcaggacctt ggagctaggc agcgaacctC aggggggcctc agaatctcag
3301 gccccaggag aggagaacct accaggagag gcagctggag gtcaggacat ggctgattcg
3361 atgctgacgc agggatctag gggcctcact gatcaggcca tattttatgc tgtgacacca
3421 ctgccctggt gtccccattc ggtggcagta tgccccatac ctgcagcagg cctagacgtg
3481 acccaacctt gtggggactg tggaacaatc caagagaact gggtgtgtct ctcttgctat
3541 caggtctacc gtggtcgtta catcaatggc cacatgctcc aacaccatgg aaattctgga
3601 cacccgctgg tcctcagcca catcgacctg tcagcctggc gttactactg tcaggcctat
3661 gtccaccacc aggctctcct agatgtgaag aacatcgccc accagaacaa gtttggggag
3721 gatatgcccc acccacacta agccccagaa tacggtccct cttcaccttc tgaggcccac
3781 gatagaccag ttccagcctg ttccaggctg taccttggat gaggggtagc ctcccactgc
3841 atcccatcct gaatatcctt tgcaactccc caagagtgct tatttaagtg ttaatacttt
3901 taagagaact gcgacgatta attgtggatc tccccctgcc catcgcccgc ttgaggggca
3961 ccactactcc agcccagaag gaaagggggg cagctcagtg gccccaagag ggagccgata
4021 tcatgaggat aacattggcg ggaggggagt taactggcag gcatggcaag gttgcatatg
4081 taataaagta caagctgtt (SEQ ID NO: 12)
```

FIG. 6B-3

SUBSTITUTE SHEET (RULE 26)

```
  1 mdlrvgqrpp vepppeptll alqrpqrlhh hlflaglqqq rsvepmrlsm dtpmpelqvg
 61 pqeqelrqll hkdkskrsav assvvkqkla evilkkqqaa lertvhpnsp gipyrtlepi
121 etegatrsml ssflppvpsi psdppehfpl rktvsepnlk lrykpkksle rrknpllrke
181 sappslrrrp aetlgdssps ssstpasgcs spndsehgpn pilgdsdrrt hptlgprgpi
241 lgsphtplfl phglepeagg clpsrlqpil lldpsgshap lltvpglgpl pfhfaqsimt
301 terlsgsglh wplsrtrsep lppsatappp pgpmqprleq lkthvqvikr sakpsekprl
361 rqipsaedle tdgggpgqvv ddglehrelg hgqpeargpa plqqhpqvii weqqrlagrl
421 prgstgdcvi lplaqgghrp lsraqsspaa pasisapepa sqarvlssse tpartlpflt
481 gliydsvmlk hqcscgdnsr hpehagriqs iwsrlqergl rsqceclrgr kasieelqsv
541 hserhvllyg tnplsrlkld ngklagiiaq rmfemlpcgg vgvdtdtiwn elhssnaarw
601 aagsvtdlaf kvasrelkng favvrppghh adhstamgfc ffnsvaiacr qlqqqskask
661 askilivdwd vhhgngtqqt fyqdpsvlyi slhrhddgnf fpgsgavdev gagsgegfnv
721 nvawaggldp pmgdpeylaa frivvmpiar efspdlvlvs agfdaaeghp aplggyhvsa
781 kcfgymtqql mnlaggavvl aleggghdlta icdaseacva allgnrvdpl seegwkqkpq
841 pqchplsggr dpgaq (SEQ ID NO:13)
```

FIG. 7A

```
   1 ataataccta ccttgcagga ccacgacagg attaagtgag gaaaaacccc catgagagtg
  61 ttttgccatt gtcaagtgag cctgagggag gctgaggggg gatcaggctg tatcatgccc
 121 ccgaggacaa actttccagt ttaccctgct ccctctctct gtccctaggc tgccccaggc
 181 cctgcgcaga cacaccaggc cctcagccgc agcccatgga cctgcgggtg ggccagcggc
 241 ccccagtgga gcccccacca gagcccacat tgctggccct gcagcgtccc cagcgcctgc
 301 accaccacct cttcctagca ggcctgcagc agcagcgctc ggtggagccc atgaggctct
 361 ccatggacac gccgacgccc gagttgcagg tgggacccca ggaacaagag ctgcggcagc
 421 ttctccacaa ggacaagagc aagcgaagtg ctgtagccag cagcgtggtc aagcagaagc
 481 tagcggaggt gattctgaaa aaacagcagg cggccctaga aagaacagtc catcccaaca
 541 gccccggcat tccctacaga accccggagc ccctggagac ggaaggagcc acccgctcca
 601 tgctcagcag ccttccgcct cctgctccca gcccgcccag tgaccccccca gagcactccc
 661 ctctgcgcaa gacagtctct gagcccaacc tgaagctgcg ccataagccc aagaagtccc
 721 cggagcggag gaagaatcca ctgctccgaa aggagagtgc gcccccccagc ccccggcggc
 781 ggcccgcaga gaccctcgga gactcctccc caagtagtag cagcacgccc gcatcagggt
 841 gcagtccccc caatgacagc gagcacggcc ccaatcccat cctgggcgac agtgaccgca
 901 ggacccatcc gactctgggc ccccgggggc caatcctggg gagcccccac actcccctct
 961 tcctgcccca tggcttggag cccgaggctg gggcaccttt gccctcccgc ctgcagccca
1021 ttcctctcct ggacccctca ggctctcatg ccccgctgct gactgtgccc gggcttgggc
1081 ccttgccctt ccactttgcc cagtccttaa tgaccaccga gcggctctct gggtcaggcc
1141 tccactggcc actgagccgg actcgctcag agcccctgcc ccccagtgcc accgctcccc
1201 caccgccggg ccccatgcag ccccgcctgg agcagctcaa aactcacgtc caggtgatca
1261 agaggtcagc caagccgagt gagaagcccc ggctgcggca gataccctcg gctgaagacc
1321 tggagacaga tggcggggga ccgggccagg tggtggacga cggcccggag cacagggagc
```

FIG. 7B-1

1381 tgggccatgg gcagcccgag gccagaggcc ccgctcctct ccagcagcac cctcaggtgt
1441 tgctctggga acagcagcga ctggctgggc ggctcccccg gggcagcacc ggggacactg
1501 tgctgcttcc tctggcccag ggtgggcacc ggcctctgtc ccgggctcag tcttccccag
1561 ccgcacctgc ctcactgtca gccccagagc ctgccagcca ggcccgagtc ctctccagct
1621 cagagacccc tgccaggacc ctgcccttca ccacagggct gatctatgac tcggtcatgc
1681 tgaagcacca gtgctcctgc ggtgacaaca gcaggcaccc ggagcacgcc ggccgcatcc
1741 agagcatctg gtcccggctg caggagcggg ggcctcggag ccagtgtgag tgtctccgag
1801 gccggaaggc ctccctggaa gagctgcagt cggtccactc tgagcggcac gtgctcctct
1861 acggcaccaa cccgctcagc cgcctcaaac tggacaacgg gaagctggca gggctcctgg
1921 cacagcggat gtttgagatg ctgccctgtg gtggggttgg ggtggacact gacaccatct
1981 ggaatgagct tcattccLcc aatgcagccc gctgggccgc tggcagtgtc actgacctcg
2041 ccttcaaagt ggcttctcgt gagctaaaga atggtttcgc tgtggtgcgg cccccaggac
2101 accatgcaga tcattcaaca gccatgggct tctgcttctt caactcagtg gccatcgcct
2161 gccggcagct gcaacagcag agcaaggcca gcaaggccag caagatcctc attgtagact
2221 gggacgtgca ccatggcaac ggcacccagc aaaccttcta ccaagacccc agtgtgctct
2281 acatctccct gcatcgccat gacgacggca acttcttccc ggggagtggg gctgtggatg
2341 aggtaggggc tggcagcggt gagggcttca atgtcaatgt ggcctgggct ggaggtctgg
2401 acccccccat gggggatcct gagtacctgg ctgctttcag gatagtcgtg acgcccatcg
2461 cccgagagtt ctctccagac ctagtcctgg tgtctgccgg atttgatgct gctgagggtc
2521 acccggcccc acLgggtggc taccatgttt ctgccaaatg ttttggatac atgacgcagc
2581 aactgatgaa cctggcagga ggcgcagtgg tgctggcctt ggagggtggc catgacctca
2641 cagccatctg tgacgcctct gaggcctgtg tggctgctct tctgggtaac agggtggatc
2701 ccctttcaga agaaggctgg aaacagaaac cccaacctca atgccactcg ctctctggag
2761 gccgtgatcc gggtgcacag taaatactgg ggctgcatgc agcgcctggc ctcctgtcca
2821 gactcctggg tgcctagagt gccaggggct gacaaagaag aagtggaggc agtgaccgca
2881 ctggcgtccc tctctgtggg catcctggct gaagataggc cctcggagca gctggtggag
2941 gaggaagaac ctatgaatct ctaaggctct ggaaccatct gcccgcccac catgcccttg
3001 ggacctggtt ctcttctaac ccctggcaat agcccccatt cctgggtctt tagagatcct
3061 gtgggcaagt agttggaacc agagaacagc ctgcctgctt tgacagttat cccagggagc
3121 gtgagaaaat c (SEQ ID NO:14)

FIG. 7B-2

```
  1 meepeepads gqslvpvyiy speyvsmcds lakipkrasm vhslieayal hkqmrivkpk
 61 vasmeematf htdaylqhlq kvsqegdddh pdsieyglgy dcpategifd yaaaiggati
121 taaqclidgm ckvainwsgg whhakkdeas gfcylndavl gilrlrrkfe rilyvdldlh
181 hgdgvedafs ftskvmtvsl hkfspgffpg tgdvsdvglg kgryysvnvp iqdgiqdeky
241 yqicesvlke vyqafnpkav vlqlgadtia gdpmcsfnmt pvgigkclky ilqwqlatli
301 lggggynlan tarcwtyltg vilgktlsse ipdbefftay gpdyvleitp scrpdrneph
361 riqqilnyik gnlkhvv (SEQ ID NO:15)
```

FIG. 8A

```
   1 gaaattcggc acgagctcgt gccgaattcg gcacgagaac ggttttaagc ggaagatgga
  61 ggagccggag gaaccggcgg acagtgggca gtcgctggtc ccggtttata tctatagtcc
 121 cgagtatgtc agtatgtgtg actccctggc caagatcccc aaacgggcca gtatggtgca
 181 ttctttgatt gaagcatatg cactgcataa gcaaatgagg atagttaagc ctaaagtggc
 241 ctccatggag gagatggcca ccttccacac tgatgcttat ctgcagcatc tccagaaggt
 301 cagccaagag ggcgatgatg atcatccgga ctccatagaa tatgggctag gttatgactg
 361 cccagccact gaagggatat ttgactatgc agcagctata ggaggggcta cgatcacagc
 421 tgcccaatgc ctgattgacg gaatgtgcaa agtagcaatc aactggtctg gagggtggca
 481 tcatgcaaag aaagatgaag catctggttt tcgttatctc aatgatgctg tcctgggaat
 541 attacgattg cgacggaaat ttgagcgtat tccctacgtg gattcggatc tgcaccatgg
 601 agatggtgta gaagacgcat tcagtttcac ctccaaagtc atgaccgtgt ccctgcacaa
 661 attctcccca ggattttcc caggaacagg tgacgtgtcc gacgttggcc tagggaaggg
 721 acggtactac agtgtaaatg tgcccatcca ggatggcata caagatgaaa aatattacca
 781 gatctgcgaa agtgtactaa aggaagtata ccaagccttt aatcccaaag cagtggtctt
 841 acagctggga gccgacacaa tagctgggga tcccatgtgc tcctttaaca tgactccagt
 901 gggaattggc aagtgtctca agtacatccc tcaatggcag ttggcaacac tcatttcggg
 961 aggaggaggc tataaccttg ccaacacggc tcgatgctgg acatacttga ccggggtcat
1021 cctagggaaa acactatcct ctgagatccc agatcatgag tttttcacag catatggtcc
1081 tgattatgtg ctggaaatca cgccaagctg ccggccagac cgcaatgagc cccaccgaat
1141 ccaacaaatc ctcaactaca tcaaagggaa tctgaagcat gtggtctagt tgacagaaag
1201 agatcaggtt tccagagctg aggagtggtg cctataatga agacagcgtg tttatgcaag
1261 cagtttgrgg aatttgtgac tgcagggaaa atttgaaaga aattacttcc tgaaaatttc
1321 caaggggcat caagtggcag ctggcttcct ggggtgaaga ggcaggcacc ccagagtcct
1381 caactggacc taggggaaga aggagatarc ccacatttaa agttcttatt taaaaaaaca
1441 cacacacaca aatgaaattt ttaatctttg aaaattattt ttaagcgaat tggggagggg
1501 agtattttaa tcatcttaaa tgaaacagat cagaagctgg atgagagcag tcaccagttt
1561 gtagggcagg aggcagctga caggcagggn tngggcctcn ggaccancca ngtggagccc
1621 tgggagagan ggtactgatc ngcagactgg gagg (SEQ ID NO:16)
```

FIG. 8B

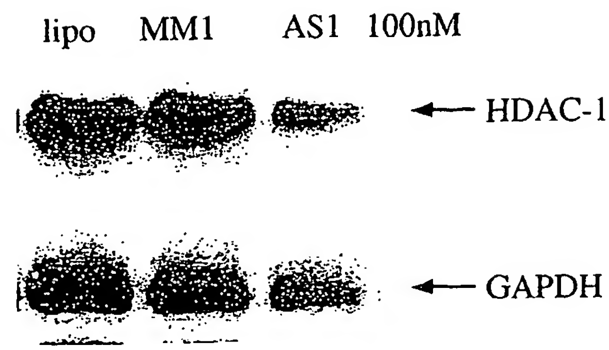


FIG. 9A

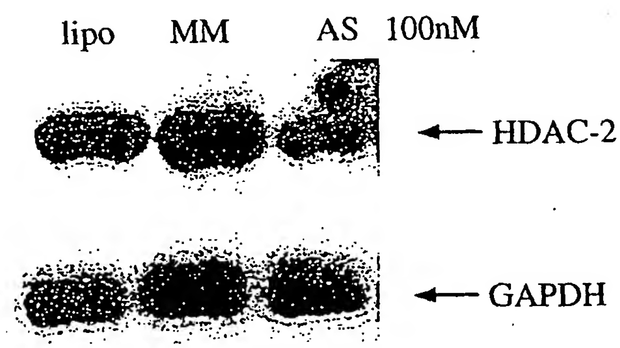


FIG. 9B

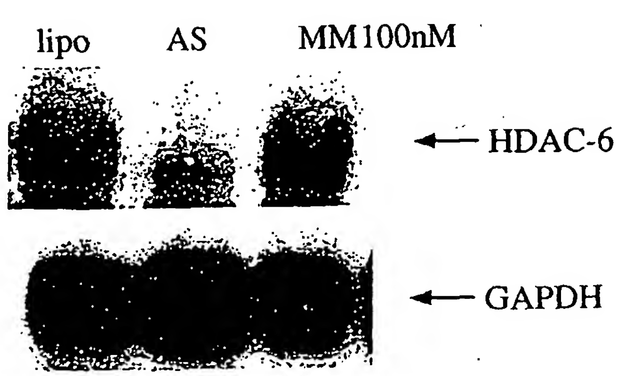


FIG. 9C

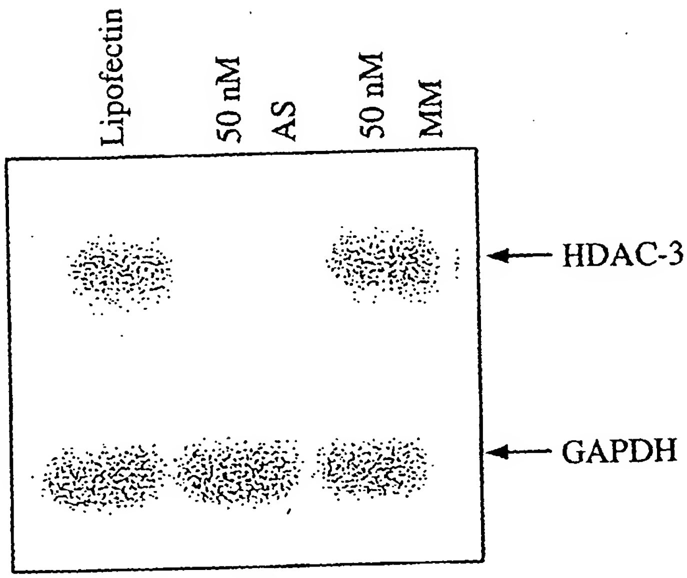

Lipofectin
50 nM AS
50 nM MM
HDAC-3
GAPDH

FIG. 9D

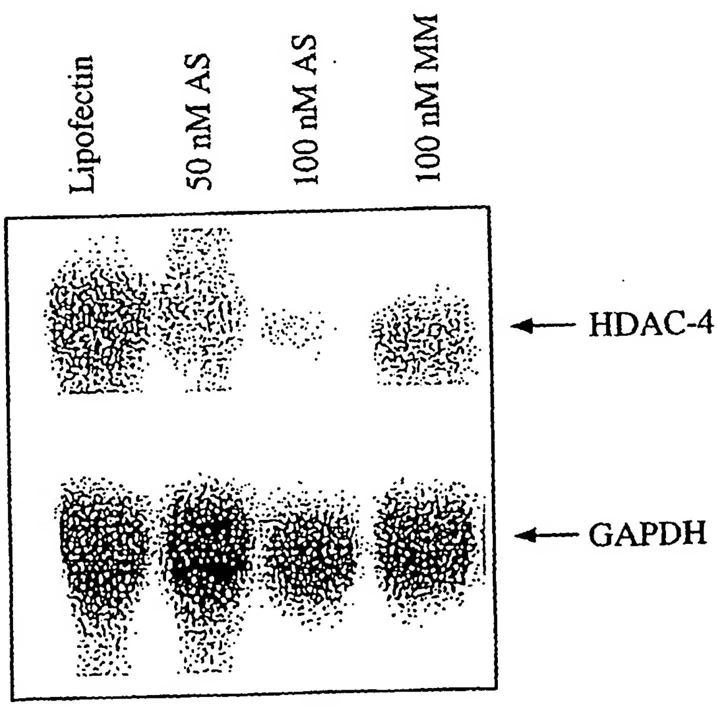

Lipofectin
50 nM AS
100 nM AS
100 nM MM
HDAC-4
GAPDH

FIG. 9E

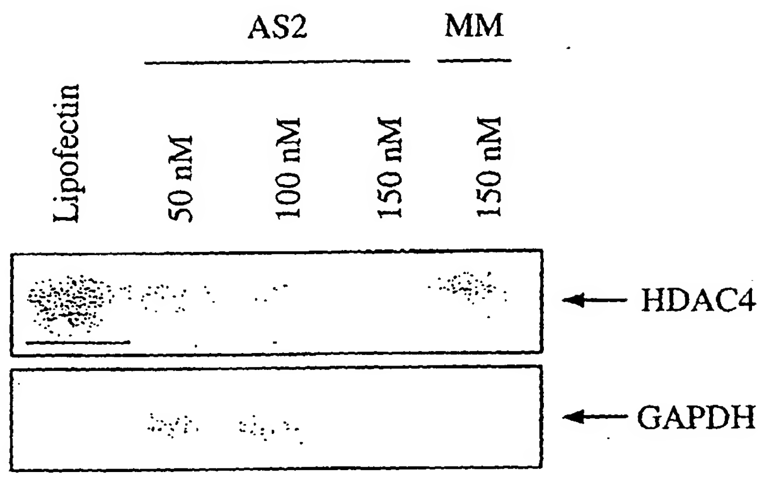


FIG. 9F

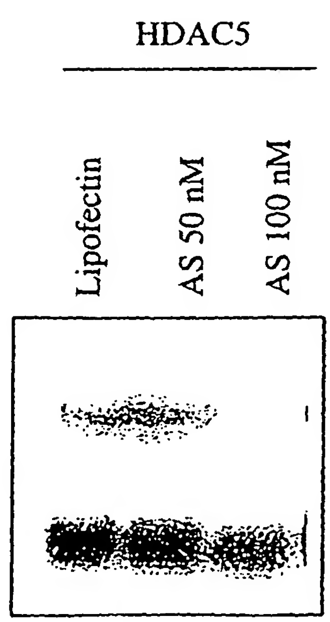


FIG. 9G

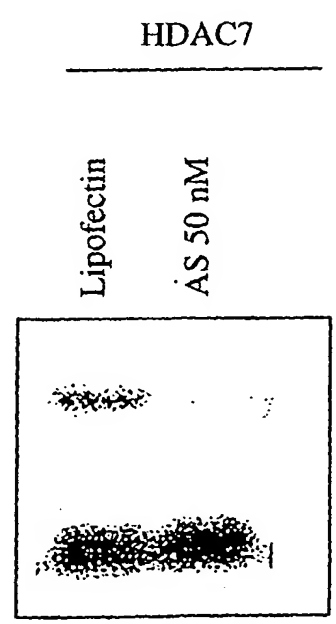


FIG. 9H

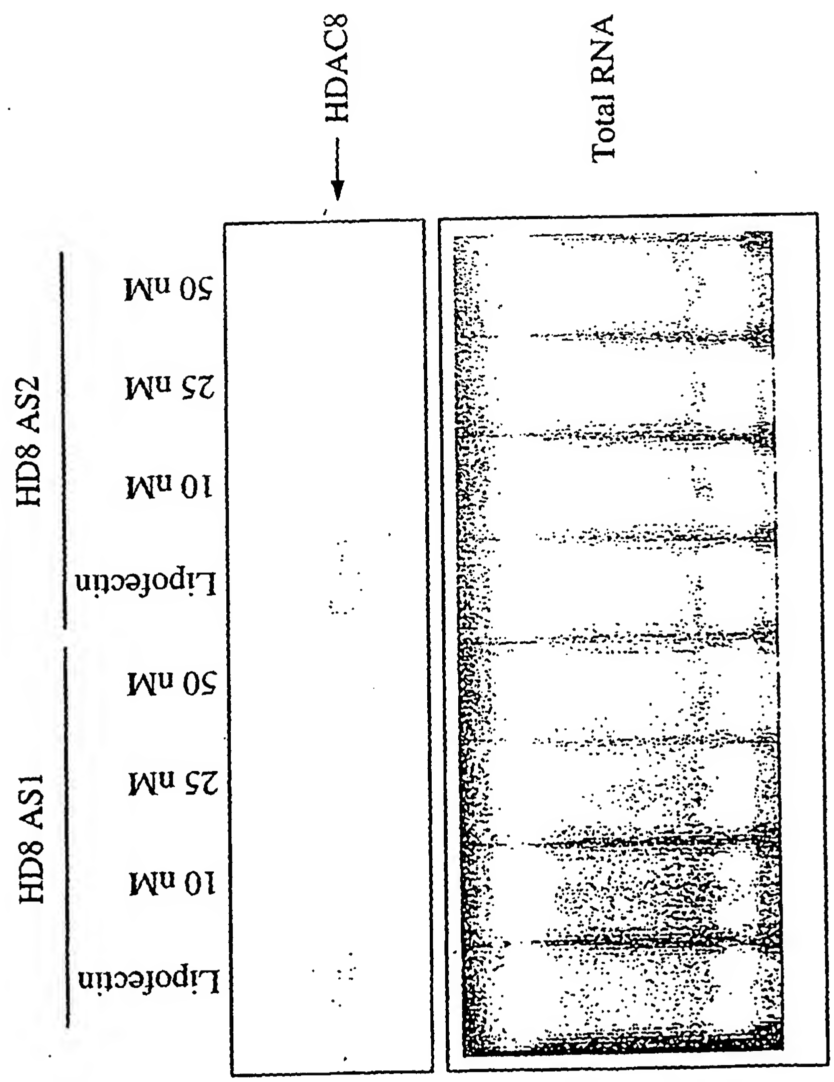
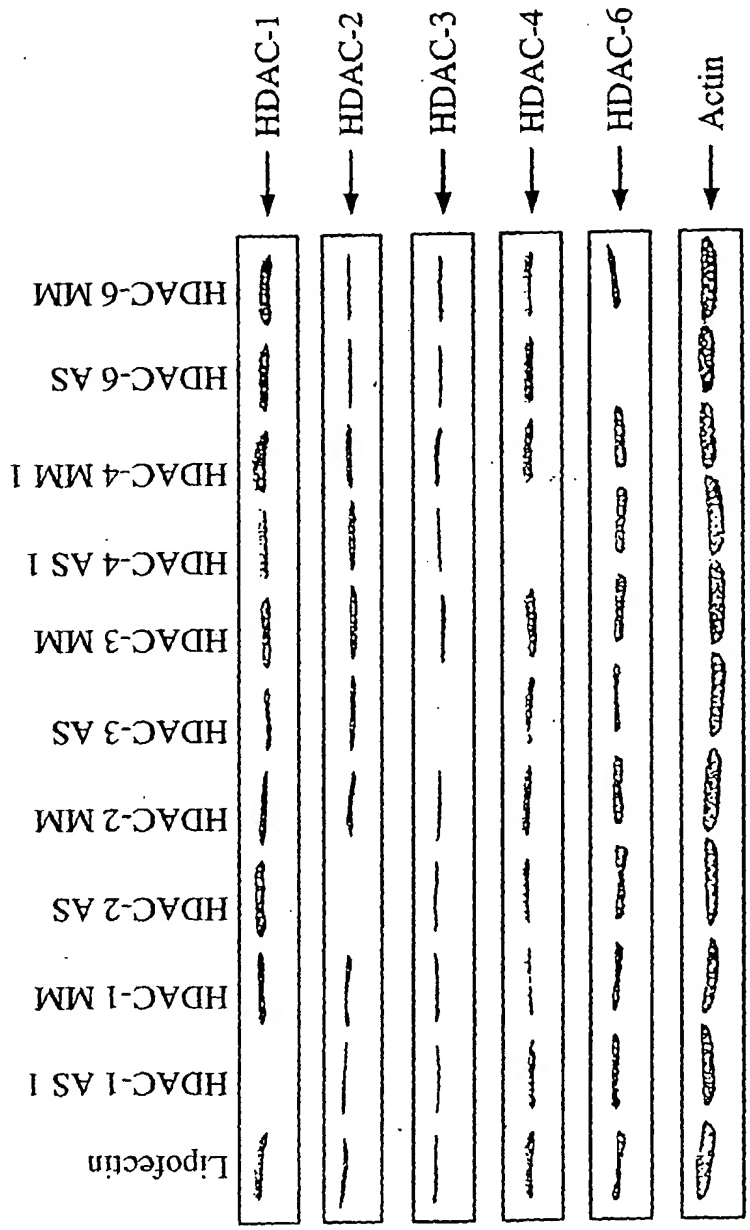

HDAC8
Total RNA
HD8 AS1
Lipofectin
10 nM
25 nM
50 nM
HD8 AS2
Lipofectin
10 nM
25 nM
50 nM

FIG. 9I



AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc – 50nM

FIG. 10A

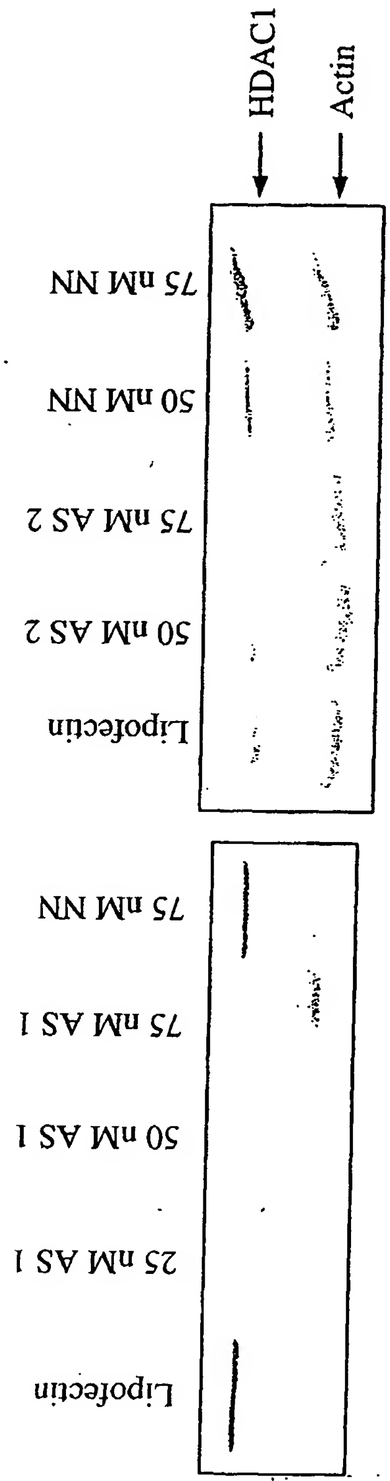

HDAC1
Actin
75 nM NN
50 nM NN
75 nM AS 2
50 nM AS 2
Lipofectin
75 nM NN
75 nM AS 1
50 nM AS 1
25 nM AS 1
Lipofectin

FIG. 10B

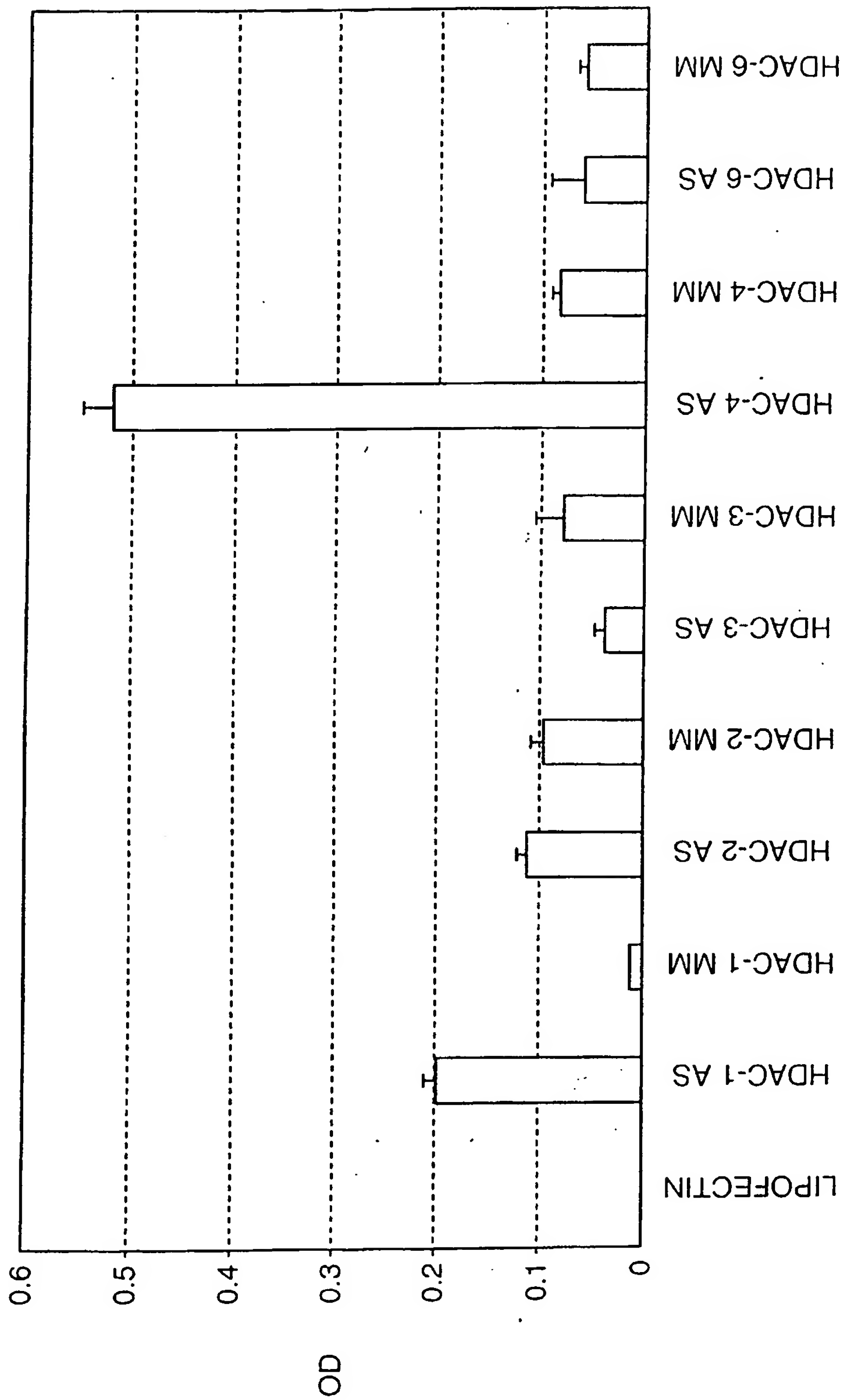

OD
0
0.1
0.2
0.3
0.4
0.5
0.6
LIPOFECTIN
HDAC-1 AS
HDAC-1 MM
HDAC-2 AS
HDAC-2 MM
HDAC-3 AS
HDAC-3 MM
HDAC-4 AS
HDAC-4 MM
HDAC-6 AS
HDAC-6 MM

FIG. 11

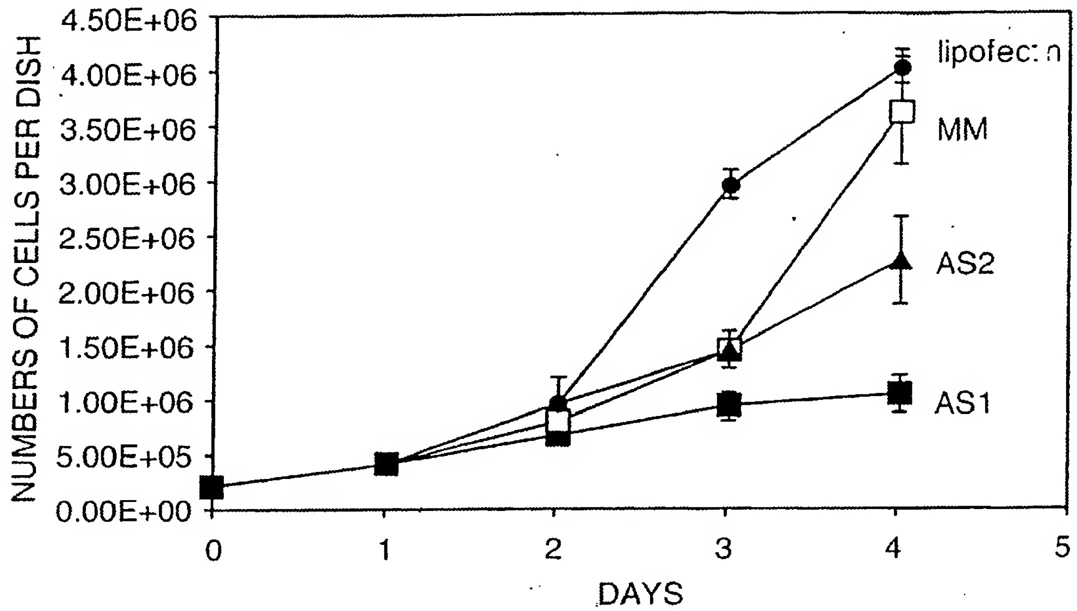


FIG. 12A

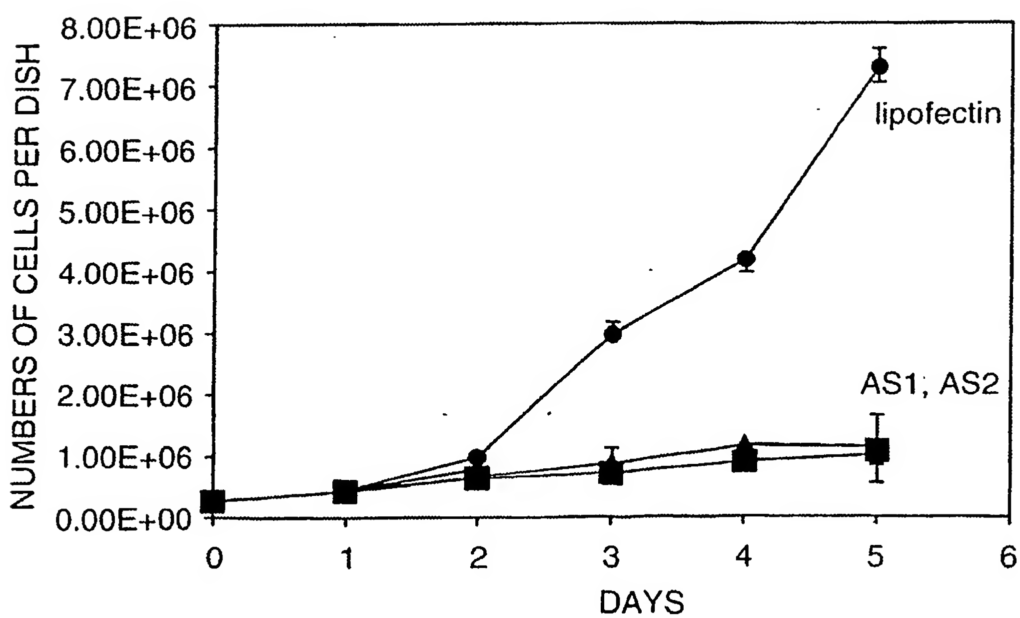


FIG. 12B

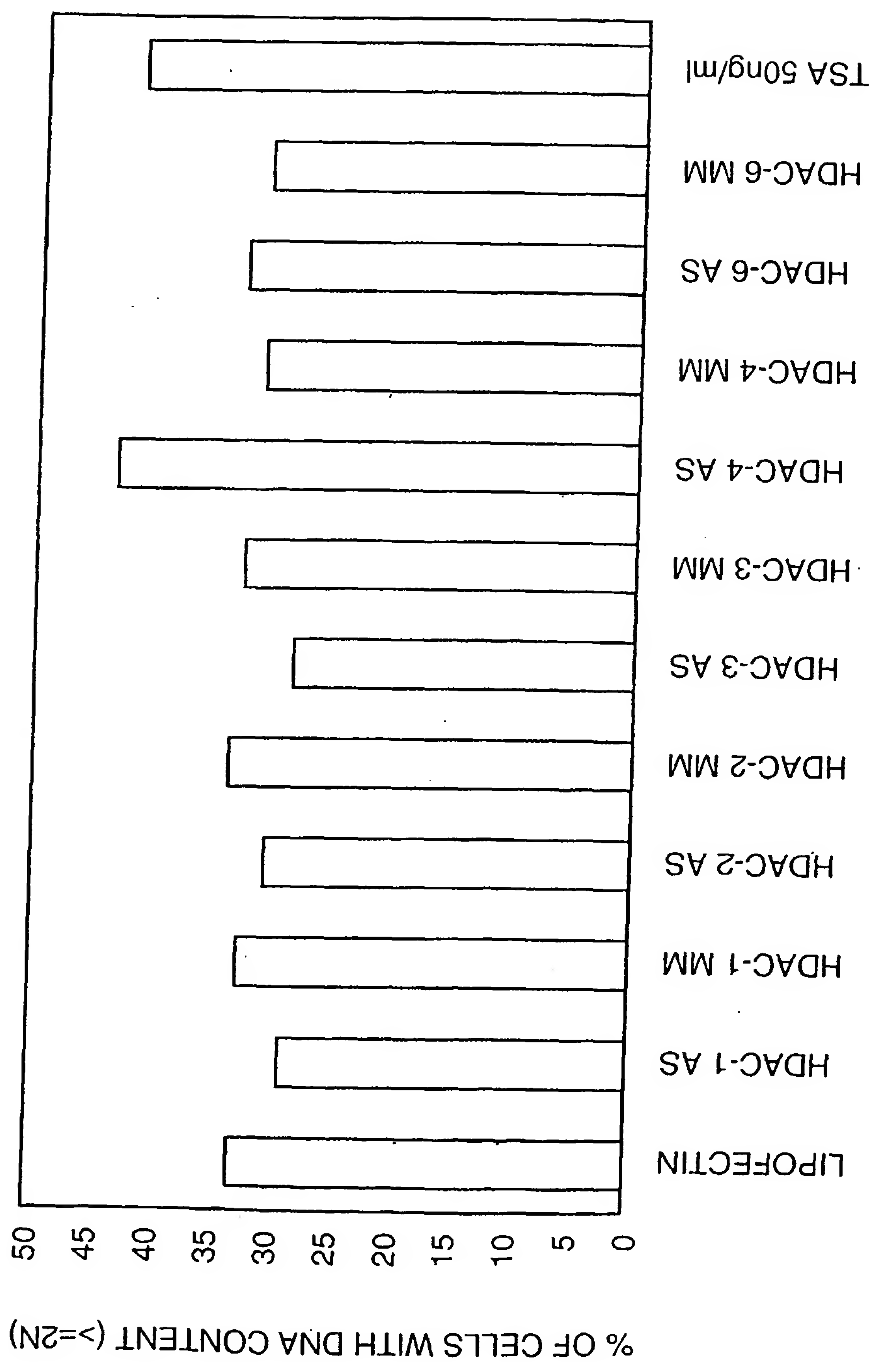

TSA 50ng/ml
HDAC-6 MM
HDAC-6 AS
HDAC-4 MM
HDAC-4 AS
HDAC-3 MM
HDAC-3 AS
HDAC-2 MM
HDAC-2 AS
HDAC-1 MM
HDAC-1 AS
LIPOFECTIN
50 45 40 35 30 25 20 15 10 5 0
% OF CELLS WITH DNA CONTENT (>=2N)

FIG. 13

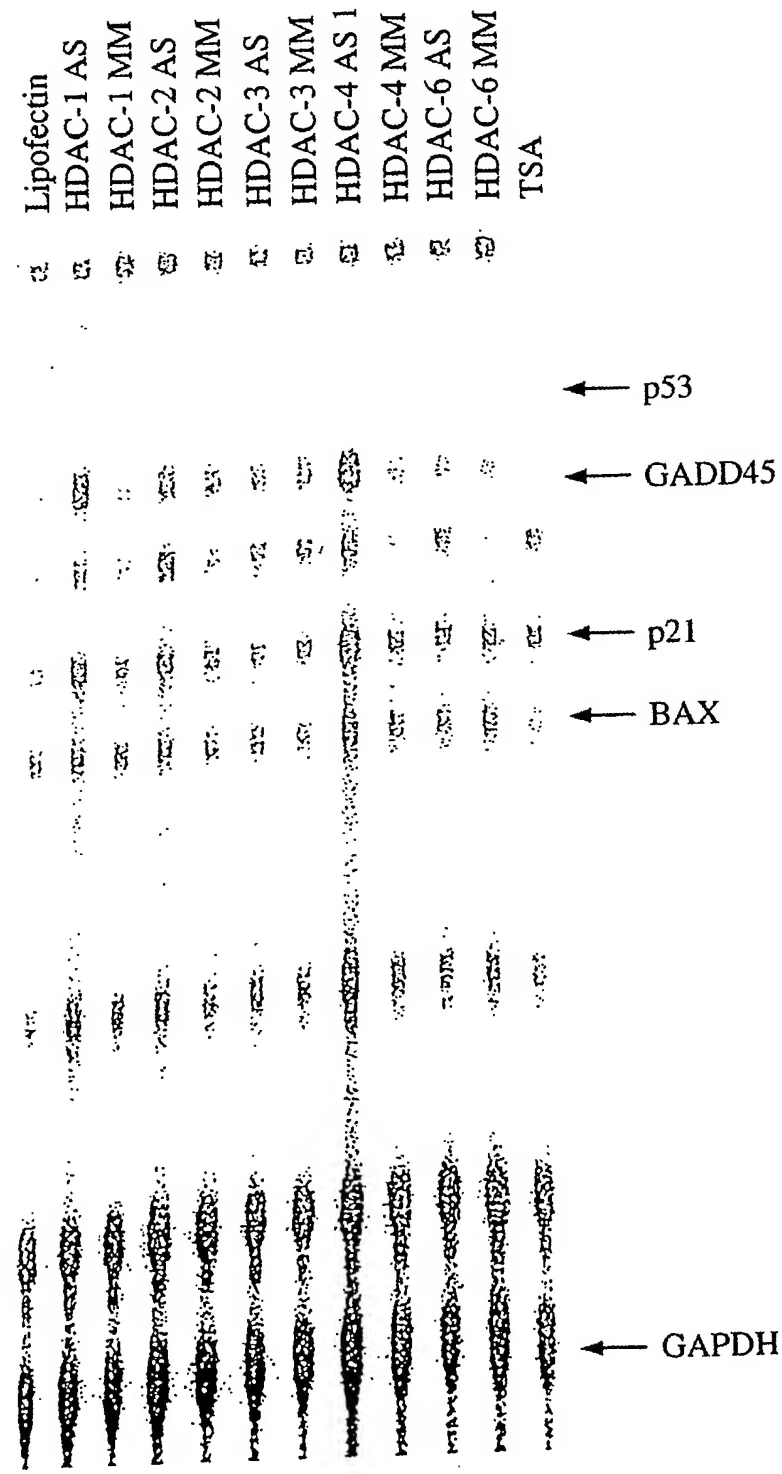

Lipofectin
HDAC-1 AS
HDAC-1 MM
HDAC-2 AS
HDAC-2 MM
HDAC-3 AS
HDAC-3 MM
HDAC-4 AS 1
HDAC-4 MM
HDAC-6 AS
HDAC-6 MM
TSA
p53
GADD45
p21
BAX
GAPDH

FIG. 14

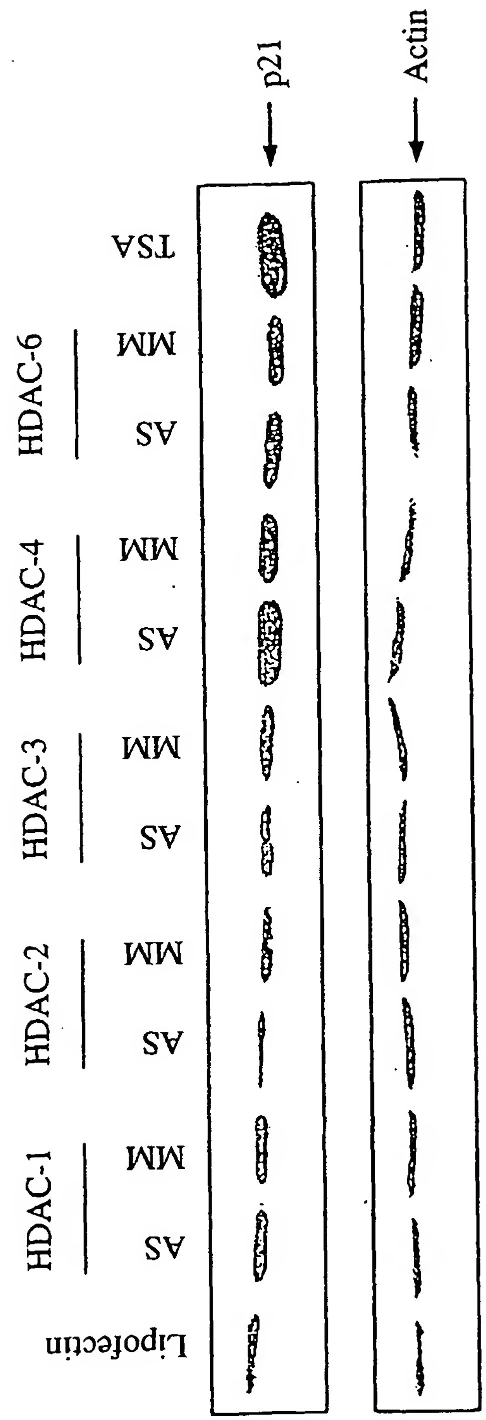

p21
Actin
TSA
HDAC-6
MM
AS
HDAC-4
MM
AS
HDAC-3
MM
AS
HDAC-2
MM
AS
HDAC-1
MM
AS
Lipofectin

FIG. 15

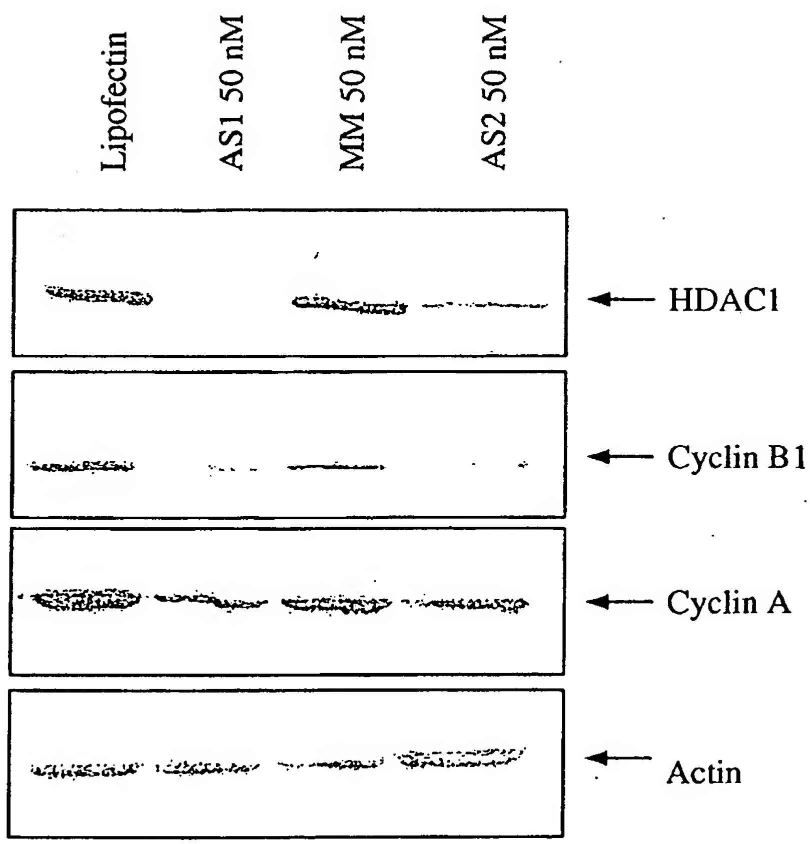

Lipofectin
AS1 50 nM
MM 50 nM
AS2 50 nM
HDAC1
Cyclin B1
Cyclin A
Actin

FIG. 16

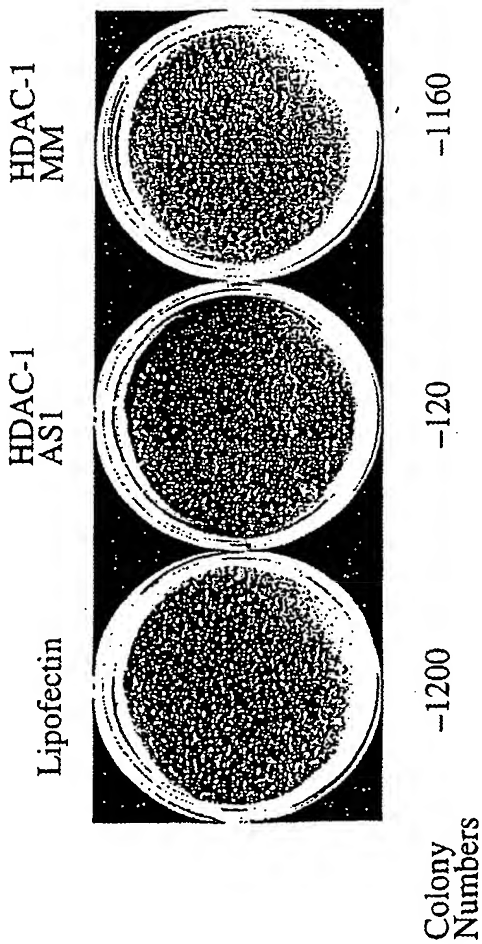


FIG. 17A

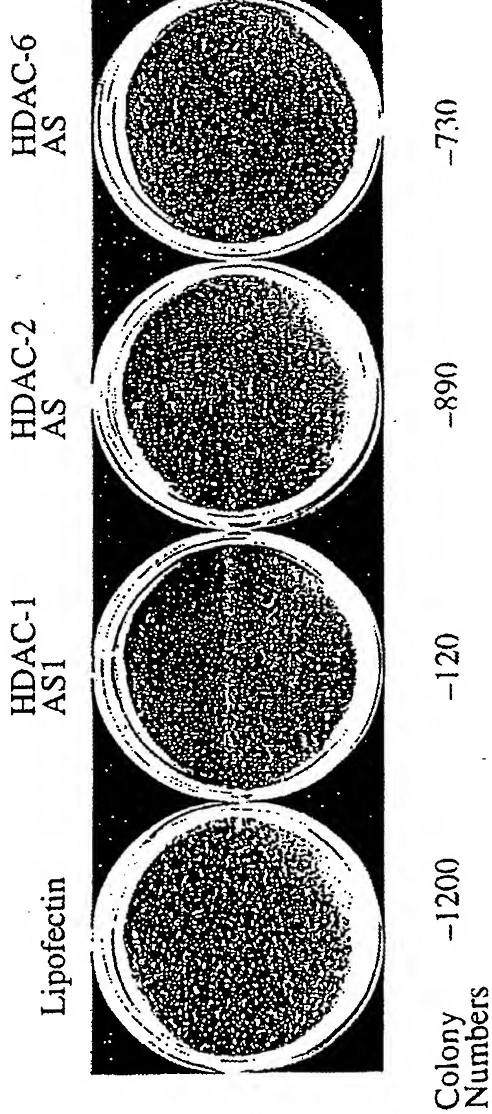


FIG. 17B

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(10) International Publication Number
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(21) International Application Number: PCT/IB2001/002907

(22) International Filing Date: 26 March 2001 (26.03.2001)

(25) Filing Language: English

(26) Publication Language: English

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60/192,157 24 March 2000 (24.03.2000) US

(71) Applicant: **METHYLGENE, INC.** [CA/CA]; 7220 Frederick-Banting, St. Laurent, Quebec H4S 2A1 (CA).

(72) Inventors: **LI, Zuomei**; 22 Oriole Street, Kirkland, H9H 3x3 (CA). **BONFILS, Claire**; 10629 Rue St. Hubert, Montreal, Quebec H9X 3V3 (CA). **BESTERMAN, Jeffrey**; 51 Gray Crescent, Baie d'Urfe, H9X 3V3 (CA).

(74) Agents: **COTE, France** et al.; Swabey Ogilvy Renault, 1981 McGill College Ave. - Suite 1600, Montréal, Québec H3A 2Y3 (CA).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 13 May 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2003/006652 A3

(54) **Title:** INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

(57) **Abstract:** This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 A61K31/7125 C07H21/04 C12Q1/44 //A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35990 A (JAMISON TIMOTHY F ;HARVARD COLLEGE (US); TAUNTON JACK (US); HASSIG) 2 October 1997 (1997-10-02) page 5, line 8 -page 6, line 27 page 27, line 13 -page 29, line 2 page 48, line 15 -page 65 claims; examples ---	1-3,6-8, 26-48

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2003	06/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YOSHIDA M ET AL: "POTENT AND SPECIFIC INHIBITION OF MAMMALIAN HISTONE DEACETYLASE BOTH IN VIVO AND IN VITRO BY TRICHOSTATIN A" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 28, 5 October 1990 (1990-10-05), pages 17174-17179, XP000616087 ISSN: 0021-9258 cited in the application the whole document	1,26,45
A	ZHAO Q ET AL: "EFFECT OF DIFFERENT CHEMICALLY MODIFIED OLIGODEOXYNUCLEOTIDES ON IMMUNE STIMULATION" BIOCHEMICAL PHARMACOLOGY, vol. 51, no. 2, 26 January 1996 (1996-01-26), pages 173-182, XP000610208 ISSN: 0006-2952 the whole document	4,5,9
P,X	WO 00 71703 A (METHYLGENE INC) 30 November 2000 (2000-11-30) the whole document	1-11, 26-48
P,X	WO 00 23112 A (BESTERMAN JEFFREY M ;MACLEOD ALAN ROBERT (CA); METHYLGENE INC (CA)) 27 April 2000 (2000-04-27) examples 9,10 page 29; tables 2,3 claims 38-50	1-12, 26-37, 44-48
E	WO 01 70675 A (METHYLGENE INC) 27 September 2001 (2001-09-27) page 46 -page 54; table 1 page 68; example 13 page 203 -page 223; examples 159-162 claims	1-16, 24-37, 44-48

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 26-33 (as far as in vivo methods are concerned) and claims 34-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 17-23
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9,26-48 (all partially) and claims 10-11

   An antisense oligonucleotide against HDAC1; modified forms thereof and its applications in therapy and diagnostic.

2. Claims: 1-9,26-47 (all partially) and claims 12-13

   As for subject 1., but concerning HDAC2.

3. Claims: 1-9,26-47 (all partially) and claims 14-15

   As for subject 1., but concerning HDAC3.

4. Claims: 1-9,26-48 (all partially) and claim 16

   As for subject 1., but concerning HDAC4.

5. Claims: 1-9,26-47 (all partially)

   As for subject 1., but concerning HDAC5.

6. Claims: 1-9,26-47 (all partially)

   As for subject 1., but concerning HDAC6.

7. Claims: 1-9,26-47 (all partially)

   As for subject 1., but concerning HDAC7.

8. Claims: 1-9,26-47 (all partially) and claims 24-25

   As for subject 1., but concerning HDAC8.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17-23

The application as filed does not comprise claims 17 to 23. Consequently only claims 1-16 and 24-48 have been taken into account.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 01/02907

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9735990 A	02-10-1997	AU 2990597 A	17-10-1997
		WO 9735990 A2	02-10-1997
WO 0071703 A	30-11-2000	AU 6718200 A	12-12-2000
		EP 1173562 A2	23-01-2002
		WO 0071703 A2	30-11-2000
		JP 2003500052 T	07-01-2003
WO 0023112 A	27-04-2000	AU 6519499 A	08-05-2000
		EP 1243289 A2	25-09-2002
		EP 1243290 A2	25-09-2002
		EP 1123111 A1	16-08-2001
		JP 2002528391 T	03-09-2002
		WO 0023112 A1	27-04-2000
WO 0170675 A	27-09-2001	AU 4870101 A	03-10-2001
		EP 1280764 A2	05-02-2003
		WO 0170675 A2	27-09-2001
		US 2002115826 A1	22-08-2002